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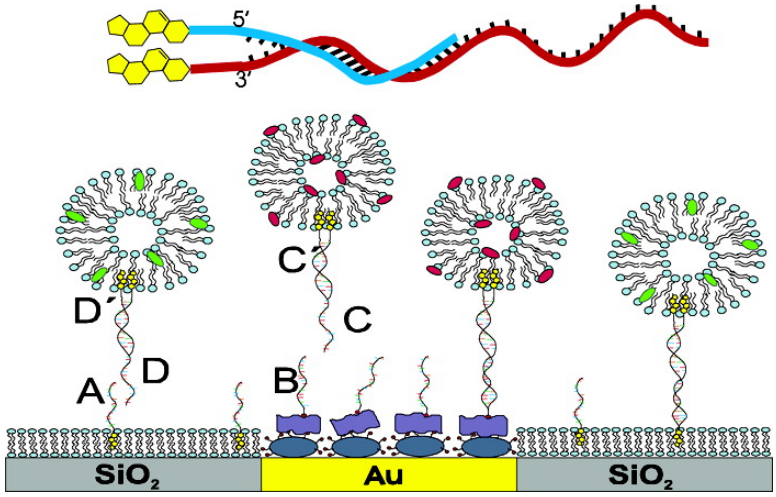

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Bivalent Cholesterol-Based Coupling of Oligonucletides to Lipid Membrane Assemblies

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Rapid progress in protein-chip technologies is today made with respect to water soluble proteins,[1] but to generate a signature of the whole proteome makeup, membrane proteins, which constitute an important group of proteins being a common target in disease diagnostics and therapeutic drugs, must also be addressable. This class of proteins is often identified as an extremely difficult group of proteins to be analyzed on this format. In fact, the first low-density protein chip based on membrane proteins was only recently reported,[2] demonstrating an array produced via microdispensing of G protein-coupled receptor containing lipid membranes. However, to fully explore the potential of array-based analysis of membrane proteins, tethered *lipid vesicles* have recently emerged as a most promising alternative, not the least since they offer the possibility to measure also membrane-protein-mediated material transport across the membrane.[3] Nevertheless, efficient means to control the positioning of *different* types of vesicles on predefined regions are still lacking. By combining the concept of DNA-labeled vesicles, previously utilized for signal enhancement of DNA hybridization detection,[4] with the concept of using DNA-labeled biomolecules for site-selective binding on cDNA arrays,[5] we[6] and others[7] recently demonstrated the use of cDNA arrays for site-selective and sequence-specific coupling of DNA-tagged lipid vesicles. Instead of using covalent coupling of DNA to chemically active lipids,[7,8] we made use of cholesterol-modified ss-DNA for spontaneous anchoring into the hydrophobic interior of lipid membranes. This means of anchoring DNA adds a threefold advantage: the method (i) is faster, (ii) does not require chemically modified lipids to be introduced, and (iii) makes use of a naturally occurring membrane constituent, thus eliminating the risk for side effects induced by chemically reactive lipid headgroups on incorporated membrane constituents. However, the cholesterol-based anchoring of DNA turns out to be relatively weak, thus complicating quantitative control of the number of DNA per vesicles. In addition, site-selective sorting of different DNA-tagged vesicles to cDNA arrays must, because of influence from DNA exchange between differently tagged vesicles, be accomplished in a sequential,[6] rather than parallel,[7] manner.

By mimicking Nature's way of utilizing multivalent interactions,[9] we herein present a novel means to improve the strength of cholesterol-based DNA coupling to lipid membranes. A bivalent cholesterol-based coupling of DNA was accomplished by hybridization between a 15-mer DNA and a 30-mer DNA, being modified with cholesterol in the 3′- and 5′-ends, respectively (Figure 1).[10] The detailed design of the construct was defined by choosing 12 bases on the 30-mer strand to be complementary to 12 bases on the 15-mer strand. Furthermore, the sequences were chosen such that the duplex formed by incubating these strands forced the two cholesterol moieties into close proximity, still separated from the duplex region by a pair of nonhybridized (3C) spacers.[10]

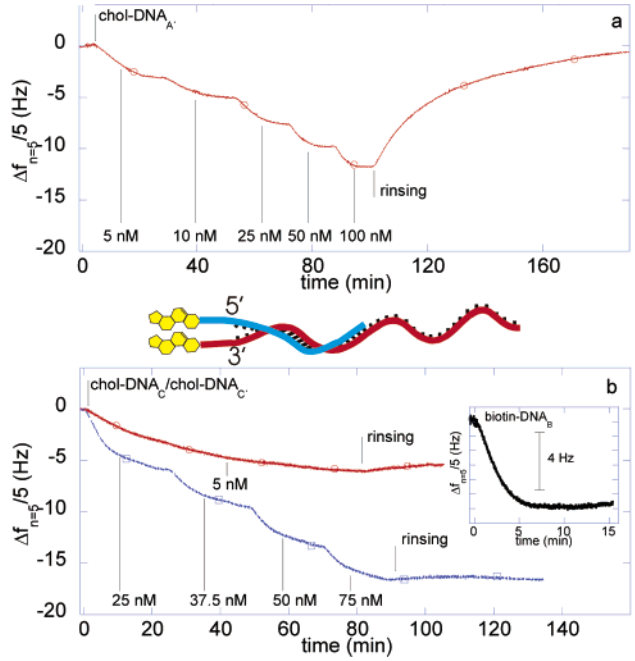

Figure 1. (a) Changes in f vs time obtained upon addition of chol-DNA$_C$ to an SPB on a SiO_2-coated QCM-D sensor surface at increasing concentrations: 5, 10, 25, 50, and 100 nM at a flow rate of 250 μL/min. (In static mode, cf. Figure 2, the desorption upon rinsing was significantly slower.) (b) The same type of data as in (a) obtained upon addition of the DNA construct comprising prehybridized (30 min incubation) chol-DNA$_C$ and chol-DNA$_{C'}$ upon increasing concentrations: 25, 37.5, 50, and 75 nM and 5 nM, only. After saturated binding, the solutions were exchanged to pure buffer II.[10] Also shown as an inset in (b) is a subsequent addition of biotin-DNA$_B$, being complementary to 15 free-hanging bases on the chol-DNA$_C$/chol-DNA$_{C'}$ duplex construct.

Figure 1a shows changes in f (cf. coupled mass) from quartz crystal microbalance with dissipation (QCM-D) monitoring upon stepwise addition of chol-DNA$_{A'}$ at increasing concentrations to a supported phospholipid bilayer (SPB)-coated SiO_2 surface, formed as described previously.[11] Saturated binding was reached at all concentrations, and the system was thoroughly rinsed in buffer after saturated binding at 100 nM, demonstrating full reversibility. This behavior is in agreement with a Langmuir-adsorption behavior,[12] which, under the assumption that the amount of water sensed by QCM-D does not vary significantly with coverage, gives K_d ($=k_{off}/k_{on}$) and k_{off} values of 16.7 ± 4 nM and ~5.8 × 10^{-4} s^{-1}, respectively.[13] In contrast, the binding of the duplex construct (chol-DNA$_C$/chol-DNA$_{C'}$) carrying two cholesterol moieties reaches apparent equilibrium only at 5 nM (Figure 1b) and displays *irreversible* coupling independent of concentration. The lack of saturated binding, which is attributed to a coverage-dependent reorganization of immobilized DNA (to be treated elsewhere), and the irreversible coupling exclude a Langmuir-based analysis of these

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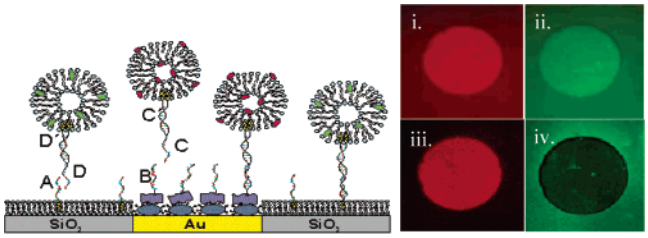

Figure 2. (Left) Schematic illustration of the DNA array produced as described previously.[6] In brief, the surface pattern was achieved via preferential adsorption of biotin-BSA (10 μg/mL) to the Au spots ($\varnothing \approx$ 250 μm), leaving the surrounding SiO_2 substrate available for spontaneous SPB formation.[11] These steps were then followed by subsequent addition of NeutrAvidin (10 μg/μL), which binds to biotin-BSA-modified Au only. Subsequent additions of biotin-DNA_B (0.1 μM) and chol-DNA_A (0.1 μM) resulted in specific binding of biotin-DNA_B and chol-DNA_A to NeutrAvidin/biotin-BSA/Au and SPB/SiO_2, respectively (A and B). (Right) Micrographs illustrating sorting of different DNA-tagged vesicles. Micrographs (i) and (ii) were obtained by exposing the DNA-modified substrate (left) for 30 min to a mixture of Rhodamine-labeled and NBD-labeled vesicles. The red and green vesicle suspensions were incubated (30 min) with monovalent chol-$DNA_{A'}$ and chol-DNA_B, respectively, prior to mixing (10 min). (iii) and (iv) shows an identical experiment, but for vesicle suspensions incubated with DNA constructs comprising chol-DNA_C/chol-$DNA_{C'}$ and chol-DNA_D/chol-$DNA_{D'}$ prior to mixing (C/C and D/D). The molar DNA vesicle ratio was 4. The images (Zeiss Axioplan 2) were analyzed with a green filter (exc. = 450−490 nm/em. = 515−565 nm) for images (ii) and (iv) and a red filter (exc. = 546 nm/em. = 590 nm) for images (i) and (ii).

data. However, the results clearly demonstrate that k_{off} is reduced by at least 1 order of magnitude compared with the monovalent case. Even if the increase in the binding strength may very well approach the theoretical value of $(1/K_d)^2$,[9] the most important observation is that the coupling is irreversible.

First, this means that the bivalent coupling can be used to precisely control the number of DNA per lipid-membrane area. Second, efficient binding upon addition of fully complementary biotin-DNA_B (inset in Figure 1b) demonstrates the feasibility of this template for detailed DNA-hybridization kinetics studies. Third, exchange of DNA between different DNA-modified vesicles is likely to be significantly reduced.

To test the latter hypothesis, the bivalent cholesterol coupling was tested by producing biotin-DNA_B-modified gold spots surrounded by a planar SPB modified with chol-DNA_A, thus comprising the simplest possible "cDNA array" (Figure 2). To evaluate parallel sorting, the "cDNA array" was exposed to suspensions containing two differently fluorescent labeled vesicles (red and green), in one case being modified with monovalent chol-$DNA_{A'}$ and chol-$DNA_{B'}$, respectively, (cf. Figure 1a) and in the other via bivalently coupled DNA constructs, carrying single-stranded regions complementary to the immobilized chol-DNA_A and biotin-DNA_B, respectively (cf. Figure 1b).[10] Indeed, the vesicle suspension containing vesicles tagged with the bivalently coupled DNA demonstrates sequence-specific and site-selective binding to the predefined regions on the surface (iii and iv in Figure 2), whereas the monovalantly modified vesicles appear to be distributed on both regions (i and ii in Figure 2). The overall lower fluorescence on the SPB substrate is attributed to a lower coverage of chol-DNA_B than biotin-DNA_A, and the weak fluorescent dots on the spot in image iv of Figure 2 is attributed to chol-DNA_A binding to unspecifically adsorbed lipid vesicles to biotin-BSA during the SPB formation process (see Figure 2 legend).

Even if the strength of the bivalent cholesterol-based coupling must not necessarily be higher than that obtained upon covalent coupling to an activated lipid headgroup, we emphasize the simplicity of the principle and its broad application areas, including a large variety of lipid assemblies, such as, for example, lipid vesicles produced by cells or formed from crude cell membranes (results to be published). Furthermore, the successful use of a DNA-modified SPB for hybridization detection under controlled flow conditions (Figure 1b) points toward an interesting template for drug−, protein−, and DNA−DNA interaction studies. This is true in particular since the DNA coverage can be precisely controlled, which is known to be critical in the case of immobilized DNA.[14,15] Finally, this way of achieving a bivalent coupling is easily extended to DNA constructs rendering multivalent interactions, thus comprising a simple model system for fundamental studies to support recent theoretical development in this field.[16]

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(10) Water was deionized and filtered (MilliQ unit, Millipore). DNA strands: 5′-TAG-TTG-TGA-CGT-ACA-CCC-CC-3′ ($DNA_{A'}$); 5′-TAT-TTC-TGA-TGT-CCA-CCC-CC-3′ ($DNA_{B'}$); 5′-TGT-ACG-TCA-CAA-CTA-CCC-CC-3′ (DNA_A); 5′-TGG-ACA-TCA-GAA-ATA-CCC-CC-3′ (DNA_B); 5′-TAG-TTG-TGA-CGT-ACA-AAG-CAG-GAG-ATC-CCC-3′ (DNA_C); 5′-TAT-TTC-TGA-TGT-CCA-AGC-CAC-GAG-TTC-CCC-3′ (DNA_D); 5′-CCC-GAT-CTC-CTG-CTT-3′ ($DNA_{C'}$); 5′-CCC-GAA-CTC-GTG-GCT-3′ ($DNA_{D'}$), derivatized at the 3′-end with biotin (biotin-DNA_B) or cholesterol (chol-$DNA_{A'}$, chol-DNA_B, chol-$DNA_{B'}$) or at the 5′-end with cholesterol (chol-DNA_C, chol-$DNA_{C'}$, chol-DNA_D, chol-$DNA_{D'}$). DNA was purchased from MedProbe, Norway, synthesized by Eurogentec, Belgium, and characterized using MALDI-TOF. Stock solutions of DNA conjugates (20 μM in buffer I: 10 mM Tris, 1 mM EDTA, pH 8.0) and proteins (biotin-labeled BSA (Sigma, 1 mg/mL in water), neutravidin (Pierce, 1 mg/mL in buffer II: 10 mM Tris, pH 8.0, 100 mM NaCl)) were aliquoted and stored at −20 °C. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, Avanti Polar Lipids, AL) was dissolved in chloroform. For fluorescent vesicles, 0.5% (w/w) of Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (rhodamine-DHPE; exc. = 550 nm/em. = 590 nm) (Molecular Probes) or 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-HPC; exc. = 460 nm/em. = 550 nm) (Molecular Probes) was added to the lipid solution. Lipid vesicles were prepared by evaporation of the solvent under N_2 (>1 h), followed by hydration in buffer (5 mg/mL) and extrusion through 0.1 and 0.03 μm polycarbonate membranes 11× each (Whatman), stored at 4 °C under N_2. The vesicle diameter was estimated to be ∼40 nm using dynamic light scattering. DNA labeling was achieved by addition of 0.5% (w/w) of chol-DNA to the vesicle solution, corresponding to ∼4 DNA per vesicle. All experiments were made by dissolving the stock solutions in buffer II to given concentrations. Substrates (AT-cut quartz crystals, f_0 = 5 MHz, with either gold or SiO_2) and the QCM-D instrument (Q-sense D 300) were from Q-sense AB, Sweden. The crystals were cleaned in 10 mM SDS (>15 min), followed by rinsing twice with water, drying (N_2), and treating with UV ozone (10 min). SiO_2-coated crystals were patterned by evaporation of 3 nm of Ti and 100 nm of Au through a mask.
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(12) Because water entrapped in the DNA film is sensed by QCM (see, for example, ref 14), changes in f cannot be used to quantify the *amount* of coupled mass, but they were used for a relative comparison of coupled mass vs concentration only.
(13) Addition of the 30-mer chol-DNA_C displays kinetics similar to that of chol-$DNA_{A'}$ and chol-$DNA_{B'}$ (not shown).
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