water, desalted with a NAP5 column (Amersham Pharmacia, USA) according to manufacturer's protocols, and stored as working stock solutions at −20 °C until use. Epoxy-derivatized slides were prepared from plain glass slides (Sigma, USA) as previously described.[9] *N*-hydroxysuccinimide slides were also used to spot the proteins but consistently gave inferior results. Proteins were prepared in $NaHCO_3$ buffer (0.1 M, pH 9) and arrayed on epoxy slides with a spacing of 180 μm between the spots by using an statistical microarray analysis arrayer (Engineering Services Inc., Ontario, Canada). After a 2-hour incubation period the slides were either used immediately, or stored for future use at 4 °C. The slides, if stored, were typically used within 48 h of printing.

Unless otherwise indicated, probing and reactions on slides were performed as follows: Before use, the slides were quenched by treatment with phosphate-buffered saline (PBS) and glycine (0.5 M) on a shaker for 10 min. The slides were blocked with PBS, glycine (0.5 M), and bovine serum albumin (BSA; 1% w/v) for 20 min, then washed with distilled water and air dried. The labeled probe was then applied: a mixture containing the probe (2 μM) was prepared by adding stock probe solution (0.5 μL, 200 μM) to tris(hydroxymethyl)-aminomethane (Tris) buffer (49 μL, 50 mM, pH 8), and BSA (0.5 μL, 1% w/v). The resulting mixture was applied to each slide by the coverslip method[9] and incubated for 30 min in the dark. The excess probe was washed off after incubation with distilled water, and the slides were subsequently washed with PBS that contained Tween (0.2% v/v) for 15 minutes on a shaker. The slides were then washed with distilled water, air dried, and scanned with an ArrayWorx microarray scanner (Applied Precision, USA) at 548/595 nm. For the PMSF experiment, each slide was first incubated with freshly prepared PMSF (50 μL, 1 mM in 50 mM Tris, pH 8) for 30 minutes, rinsed extensively with distilled water to remove any free residual PMSF, and screened with FP-Cy3. The inhibition experiments were identical to the probe–enzyme reactions, except that varying concentrations of trypsin inhibitor (original concentration $\sim 5\ mg\,mL^{-1}$) were added to the reaction mixture together with the probe.

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Patterns of DNA-Labeled and scFv-Antibody-Carrying Lipid Vesicles Directed by Material-Specific Immobilization of DNA and Supported Lipid Bilayer Formation on an Au/SiO_2 Template

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DNA · protein chips · supported lipid bilayers · surface patterning · vesicles

Much effort is currently concentrated on research devoted to biofunctional patterned surfaces, which constitute the fundament for the development of microarrays for high-throughput gene and protein analyses. DNA microarrays have proved very successful,[1] and the concept is in the process of being applied to protein arrays.[2] However, in contrast to DNA fragments, proteins are easily denatured in contact with solid supports, and robotic printing of proteins onto chemically reactive glass slides[3] will not necessarily be applicable as a generic protocol for the preparation of protein arrays. Supported phosphatidylcholine lipid bilayers have emerged as interesting candidate substrates for protein chips, since they efficiently reduce non-specific protein adsorption[4, 5] and, at the same time, allow different strategies for protein immobilization with biospecific

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interactions; for example: biotin – streptavidin,[6, 7] Ni^{2+}-mediated binding of histidine-tagged proteins to nitrilotriacetic acid (NTA)-lipids,[8] or covalent coupling reactions such as those between maleimides and thiols.[9] Furthermore, progress in patterning of lipid bilayers and/or vesicles with varying lipid composition has been achieved by using dispensing,[10–12] micro contact printing,[13–15] gradients in microfluidic flow devices,[16–18] and/or immobilization of biotin-functionalized vesicles to streptavidin-patterned surfaces.[19] In the present work, we combine the concept of micropatterns of supported lipid bilayers with the concept introduced by Niemeyer,[20] who advocates the use of immobilized complementary DNA (cDNA) strands to direct spatial distribution of DNA-labeled proteins. Previously, DNA-modified vesicles have been used for signal enhancement of DNA hybridization reactions.[21] Here, we present the first case of *DNA-directed* immobilization of intact vesicles to patterned surfaces (arrays), where the vesicles have the in-built potential to act as *protein carriers* (for transmembrane or water-soluble proteins). The developed multistep surface modification protocol is schematically illustrated in Figure 1. The strategy was proven by detecting antigens by using histidine-tagged single-chain antibody fragments (scFv)[22] coupled to Ni^{2+} – NTA- and DNA-modified lipid vesicles, and a simple array was demonstrated by using DNA-directed immobilization of intact vesicles to two different cDNA-functionalized spots. The surface modification was experimentally verified with the quartz crystal microbalance with dissipation monitoring (QCM-D) technique[23] and fluorescence microscopy.

The conditions for the first step of the described protocol were chosen such that adsorption of biotinylated bovine serum albumin (biotin – BSA) was efficiently reduced on SiO_2 ($|\Delta f| < 2$ Hz), but pronounced on Au ($|\Delta f| > 45$ Hz; Figure 2). Subsequent addition of lipid vesicles (step ii) resulted in efficient formation of a supported lipid bilayer on SiO_2 and weak adsorption of intact vesicles on Au. From the QCM-D data in Figure 2, this was verified from the presence and the lack of the typical signature for bilayer formation on SiO_2 and Au,[24] respectively. The mass uptake of nonspecific binding of intact vesicles on Au was one order of magnitude lower than for typical adsorption of intact vesicles on bare surfaces.[25] The inert properties of the

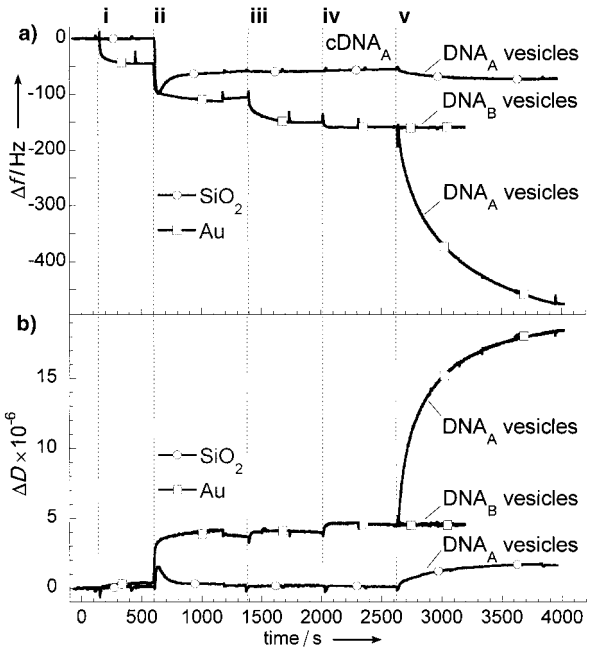

Figure 2. *QCM-D results: (a) $\Delta f_{n=3}$ related to mass uptake and (b) $\Delta D_{n=3}$ related to viscoelastic properties, from experiments establishing the surface modification protocol by using differently coated quartz crystals (SiO_2 or Au). The two surfaces were exposed to (i) biotin – BSA (10 $\mu g\,mL^{-1}$), (ii) POPC vesicles (20 $\mu g\,mL^{-1}$), (iii) neutravidin (10 $\mu g\,mL^{-1}$), (iv) biotin – $cDNA_A$ (0.25 μM), and (v) and POPC vesicles (15 $\mu g\,mL^{-1}$) doped with 0.5% cholesterol – DNA_A (see Figure 1, left-hand panel). Also shown is a superimposed (step v) addition of POPC vesicles doped with cholesterol – DNA_B, which is noncomplementary to $cDNA_A$, on Au. f = resonance frequency, D = energy dissipation.*

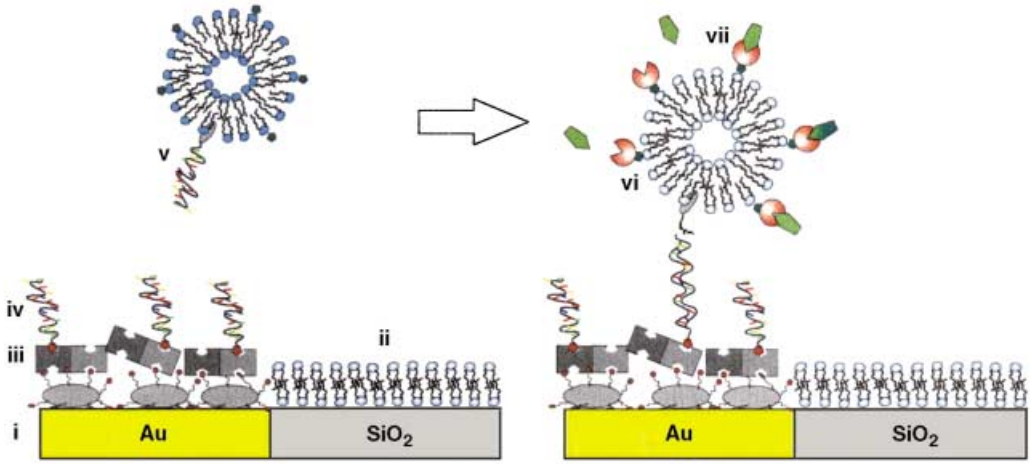

Figure 1. *Schematic representation of the substrate-directed surface modification protocol relying on (i) differences in adsorption of biotin – BSA on Au and SiO_2 and (ii) the spontaneous fusion of lipid vesicles into a supported lipid bilayer on SiO_2. (iii) Neutravidin and (iv) biotin – cDNA were subsequently bound onto the Au, whereas no binding occurred on the inert lipid bilayer on SiO_2. Eventually, because supported lipid bilayers are inert not only to proteins but also to vesicles, (v) specific immobilization of intact DNA-labeled vesicles to cDNA strands on Au was achieved. In the more advanced protocols, steps i – v were followed by (vi) scFv binding to Ni^{2+} – NTA-modified vesicles and (vii) fluorescent detection of the cholera toxin β-subunit antigen (Figure 3c), or they were made such that differently labeled vesicles were immobilized onto different cDNA-modified Au spots (Figure 3d).*

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supported lipid bilayer formed on SiO_2 then ensured preferential binding of neutravidin (step iii) to biotin–BSA on Au ($|\Delta f|_{Au} \approx 45$ Hz, $|\Delta f|_{SiO_2} < 2$ Hz), which was followed by efficient binding of biotin–$cDNA_A$ (step iv) to the layer of neutravidin ($|\Delta f|_{Au} \approx 12$ Hz, $|\Delta f|_{SiO_2} < 1$ Hz; Figure 2). To achieve, eventually, cDNA-directed coupling of intact vesicles, lipid vesicles were modified by addition of DNA–cholesterol conjugates (0.5% (w/w)) to the vesicle solution. Addition of these DNA-modified vesicles (step v) resulted in a mass uptake ($|\Delta f|$) that was 15 times greater on Au than on SiO_2; this illustrates the preferential coupling to the cDNA-modified Au rather than the SiO_2 modified with the lipid bilayer (Figure 2). The larger relative increase in D on SiO_2 compared to that on Au is attributed to a higher viscosity of vesicles at low coverage. The high ratio between the changes in ΔD and Δf further verified coupling of intact vesicles, rather than free cholesterol–DNA, thus demonstrating that the cholesterol moiety is efficiently anchored to the hydrophobic part of the lipid membrane (on average, three DNA strands per vesicle). In comparison with previous strategies developed for DNA-labeling of intact vesicles by using thiol-reactive maleimido-lipids and thiol variants of DNA,[21] the present strategy is rapid (minutes rather than hours) and more general since it does not require the introduction of chemically modified lipids but is designed for any lipid bilayer (data not yet published).

To check that the coupling of DNA-labeled vesicles relied on sequence-specific DNA hybridization, vesicles modified with DNA_B (noncomplementary to $cDNA_A$) were added to $cDNA_A$-modified Au (step v, Figure 2). Significantly, no mass uptake was observed, which proves that sequence-specific coupling of intact vesicles occurs, a prerequisite for an extension of the concept towards large-scale arrays. The surface modification protocol was repeated on a patterned substrate (Au spots evaporated on SiO_2), and the binding of differently dyed DNA-modified vesicles was imaged by fluorescence microscopy. Figure 3a shows a fluorescence micrograph verifying material specific immobilization of DNA-labeled rhodamine-dyed vesicles coupled to cDNA-modified Au spots through the sequence of additions presented in Figure 2. Very low background fluorescence was observed on the surrounding lipid-bilayer-modified SiO_2. Figure 3b shows an interesting extension of this protocol in which the lipid bilayer does not only act as an inert surrounding, but is itself functionalized with DNA. This was done by anchoring of a second cholesterol–DNA conjugate ($cDNA_B$) to the lipid bilayer on SiO_2, followed by hybridization with complementary, fluorescein-labeled DNA_B (also applicable to DNA_B-modified vesicles). Clearly, to ensure that the cholesterol–$cDNA_B$ was anchored to the supported lipid bilayer and not to vesicles coupled to the Au spot, the anchoring to the lipid bilayer was made prior to the addition of the DNA-labeled vesicles.[26] Figure 3c illustrates the extension of the concept to patterns of protein-carrying DNA-labeled vesicles. DNA_A-labeled Ni^{2+}–NTA-modified vesicles were used, which, when specifically immobilized to the $cDNA_A$-modified Au spots, allowed coupling of histidine-tagged scFv (step vi, Figure 1). This in turn allowed specific antigen (cholera toxin subunit β (CT-β)) binding (step vii, Figure 1). The fluorescence micrograph nicely demonstrates highly specific and sensitive detection of CT-β with low background fluorescence, a prerequisite for larger scale protein identification, even if minor, but still specific, binding of CT-β can be observed to intact vesicles coexisting with the supported lipid bilayer (Figure 2). Finally, Figure 3d shows an extension of the protocol towards arrays of *different* immobilized vesicles on different Au spots, thereby demonstrating the potential of the protocol for surface-directed sorting of differently functionalized vesicles. Even if these injections are presently made sequentially (see figure legend), the previous results (Figures 2 and 3b) demonstrate negligible nonspecific binding of DNA-modified vesicles, which in turn ensures that the sequence of addition of biotin–DNA and vesicles will not influence the outcome of the experiment. However, a true sorting experiment from a mixture of two or more differently DNA-labeled vesicles is, for the present protocol, likely to be efficient only at short incubation times, a problem attributed to weak, but not negligible, water

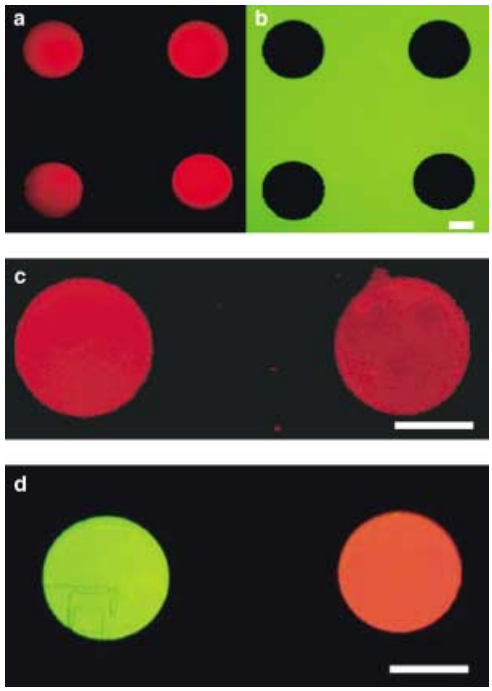

Figure 3. *Micrograph of a SiO_2-coated QCM crystal with spots of Au exposed to the sequence of injections described in Figure 2, with the exception that in (a) the vesicles were labeled with rhodamine–lipids (exc. ≈ 550 nm/em. > 590 nm) and in (b) that functionalization of the lipid bilayer was demonstrated by using cholesterol–$cDNA_B$ (25 nM) hybridized with fluorescein–DNA_B (0.25 μM; exc. ≈ 470 nm/em. ≈ 540 nm). (c) Verification of highly specific binding of Cy3-labeled cholera toxin β subunit (0.10 μM; ≈ 100 amol per spot, estimated from QCM-D data) after binding of scFv (0.25 μM) to DNA-labeled Ni^{2+}–NTA-functionalized vesicles coupled to the Au spots. Low nonspecific binding was proven by QCM-D (not shown). d) Steps i–iv (Figure 2) on the patterned substrate were followed by injection of biotin–$cDNA_B$ (20 μM) through a thin capillary, leading to preferential binding to a single Au spot (to the right). After removing the capillary, NBD-dyed (exc. ≈ 460 nm/em. ≈ 550 nm) POPC vesicles doped with cholesterol–DNA_B were bound selectively from bulk to the $cDNA_B$-modified spot (15 μg mL^{-1}, > 15 s). An adjacent spot (to the left) was thereafter functionalized by biotin–$cDNA_A$, added in excess (0.25 μM, 5 s), after which rhodamine-dyed POPC vesicles doped with cholesterol–DNA_A (15 μg mL^{-1}, > 15 s) were bound selectively to the $cDNA_A$-modified spot. Scale bars: 100 μm. NBD = 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl.*

solubility of cholesterol – DNA. This, in turn, may result in exchange of cholesterol – DNA between different vesicles in a mixture, a complication that is currently under detailed investigation and improvement.

In conclusion, we present a simple, surface-preparation protocol for DNA-directed coupling of intact vesicles that eliminates the need for dispensing or microfluidics during the critical vesicle-immobilization step. By introducing a cholesterol-based strategy for DNA-labeling of vesicles and/or supported lipid bilayers, multifunctional patterns of lipid assemblies on top of a protein/lipid bilayer template were demonstrated (Figure 3a, b) and were proven to be compatible with highly specific and sensitive antigen detection with scFv-carrying vesicles (Figure 3c). Surface-directed sorting of vesicles to two different cDNA-modified spots was also demonstrated (Figure 3d).

In light of future protein-chip applications, the present protocol is compatible not only with water-soluble proteins, as proven here, but also with transmembrane proteins, where the lipid-membrane environment is extremely critical throughout the whole preparation protocol. This protocol, combined with recent developments within fluorescence imaging, opens up the use of encapsulated dyes for the detection of, for example, protein-mediated ion translocation with high sensitivity and lateral resolution. In addition, the DNA label can be used as a variable spacer, which is advantageous over previous vesicle-capture protocols that used avidin-mediated capturing of biotin-modified vesicles,[19, 27] hydrophobic moieties in dextran gels,[28] or immobilized antibodies.[29] Note that the substrates used here were kept hydrated throughout the series of modifications. There is no desire to circumvent the need for hydration in the final coupling of intact, functionalized vesicles. However, recent progress demonstrating stabilized supported lipid bilayers that sustain drying without loosing their inertness towards protein adsorption,[30] combined with robotic printing of DNA or the use of commercial DNA chips, points towards an expansion of the initial steps (i – iv in Figure 1, 2) of this protocol for use outside the controlled laboratory conditions.

Experimental Section

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′ ($cDNA_A$); 5′-TGG-ACA-TCA-GAA-ATA-CCC-CC-3′ ($cDNA_B$)) were derivatized at the 3′-end with biotin (biotin – DNA_A, biotin – DNA_B), cholesterol (cholesterol – $cDNA_A$, cholesterol – DNA_B, cholesterol – $cDNA_B$), or fluorescein (fluorescein – $cDNA_B$) (MedProbe, Norway). Stock solutions of DNA conjugates (20 μM in 10 mM tris(hydroxymethyl)aminomethane (Tris) and 1 mM ethylenediaminetetraacetate (EDTA), pH 8.0) and proteins (biotin-labeled BSA (Sigma, 1 mg mL^{-1} in water), neutravidin (Pierce, 1 mg mL^{-1} in buffer (10 mM Tris, 100 mM NaCl, pH 8.0)) were aliquoted and stored at −20 °C. Histidine-tagged scFv (clone CT-17, 1 mg mL^{-1} in phosphate-buffered saline, pH 7.4), kindly provided by BioInvent Therapeutics (Lund, Sweden), and β-subunit cholera toxin (Sigma, St Louis, USA) labeled with Cy3 (Amersham Pharmacia Biotech, Uppsala, Sweden) were stored at 4 °C. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC; Avanti Polar Lipids, AL, USA) was dissolved in chloroform. For fluorescent vesicles, 0.5% (w/w) of Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (rhodamine-DHPE; Molecular Probes, USA) or 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-HPC; Molecular Probes, USA) was added to the lipid solution. For Ni^{2+} – NTA-modified vesicles, 5% (w/w) of 1,2-dioleoyl-*sn*-glycero-3-{[*N*-(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl} ammonium salt (DOGS-NTA; Avanti Polar Lipids, AL, USA) was added to the lipid solution. Lipid vesicles (stored at 4 °C under N_2) were prepared by evaporation of the solvent under N_2 (> 1 h), followed by hydration in buffer (5 mg mL^{-1}) and extrusion through 0.1 and 0.03 μm polycarbonate membranes (11 times through each; Whatman, USA). DNA labeling was achieved by addition of 0.5% (w/w) of cholesterol – DNA to the vesicle solution, with 250 mM imidazole present for the Ni^{2+} – NTA-modified lipids. All substrates (AT-cut quartz crystals, $f_0 = 5$ MHz, with either Au 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 sodium dodecylsulfate (> 15 s), rinsed twice with water, dried (N_2), and subjected to UV – ozone treatment (10 s). 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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