Creation of a functional heterogeneous vesicle array via DNA controlled surface sorting onto a spotted microarray

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Membrane protein microarrays are expected to play a key role in the future of drug screening and discovery. The authors present a method for the creation of functional heterogeneous vesicle arrays via DNA controlled surface sorting. Complexes of streptavidin and biotinylated DNA are spotted onto a biomolecule- and cell-resistant surface of biotinylated poly(L-lysine)-*grafted*-poly(ethylene glycol). Two kinds of vesicles functionalized with either the membrane-binding protein annexin A5 or loaded with bovine serum albumin, are tagged with DNA, mixed together, and guided to predefined spots on the surface. The authors show that the spotted complexes remain active and selective and that the background is resistant towards nonspecific adsorption of the vesicles and the proteins. © *2006 American Vacuum Society.* [DOI: 10.1116/1.2434178]

I. INTRODUCTION

Microarrays are primarily used for the parallel screening of large amounts of target molecules. DNA microarrays[1–3] have proven to be successful; however, there remains a strong need for protein arrays. Protein microarrays have attracted interest as a tool for drug discovery and screening.[4–7] However, the current processes that are used to create the DNA microarrays (e.g., spotting or photolithography based approaches) cannot be directly applied for most proteins.[8] The main difficulty comes from the fact that most proteins need to be kept in their natural environment in order to preserve their full activity; therefore, drying during the spotting process is likely to interfere with the proteins' performance.[9] Fragile membrane proteins are especially prone to structural damage upon ambient exposure. So far only Fang *et al.* have reported on spotting membrane microarrays including G-protein coupled receptors without major loss of their activity.[5] Others have attempted to spot a linker as a first step and then add the membrane protein in order to keep them in their natural environment, i.e., in a lipid membrane which would make membrane protein microarrays more universally applicable.[10–13] The creation of heterogeneous vesicle arrays has been demonstrated by sequential immobilization,[14] by random positioning,[11] or via surface sorting from a mixture of different kinds of vesicles.[15,16] However, sequential immobilization and random positioning suffer from major drawbacks that limit their practical applicability: they are time consuming and limited in terms of size and number of spots or there is an identification problem, respectively. Controlled sorting of DNA-tagged vesicles to an array of surface bound complementary strands overcomes these two problems, but it is highly sensitive to the nonspecific adsorption of the vesicles to the background and onto the other spots. Recently we presented an approach to create a homogeneous vesicle microarray based on a micropatterning method[17] using DNA as a linker between the surface and the vesicles.[8] The micropatterning method provided spots of bioactive patches in a bioresistant background of a PEGylated graft copolymer (PLL-*g*-PEG).[18,19]

Here we report the realization of a heterogeneous array of protein functionalized vesicles using DNA as a linker with the aim of surface sorting DNA-tagged nano-objects such as vesicles. We have successfully solved the problem of nonspecific binding of the vesicles by coating the substrate with poly(L-lysine)-*grafted*-poly(ethylene oxide) with biotin functionalities[20] (PLL-*g*-PEG/PEGbiotin) onto which streptavidin complexed with the primary DNA strands was deposited using pin and ring spotting. Two kinds of vesicles each tagged with a different DNA sequence (tDNA1 and tDNA2) were mixed in solution and exposed to the microarray containing the predefined spots of complementary DNA strands. The vesicles tagged with tDNA1 have been functionalized with green labeled annexin A5 as a representative membrane-binding protein,[21–23] while the vesicles tagged with tDNA2 were loaded with red labeled bovine serum albumin (BSA) [Fig. 1(a)]. A fluorescent microarray reader was used to monitor the sorting of the vesicles to the corresponding spots of the microarray. The results not only demonstrate the solution based arraying of membrane bound proteins but they also show the feasibility of arraying proteins confined in small compartments that could be used for the high throughput study of, e.g., enzymatic reactions.[24]

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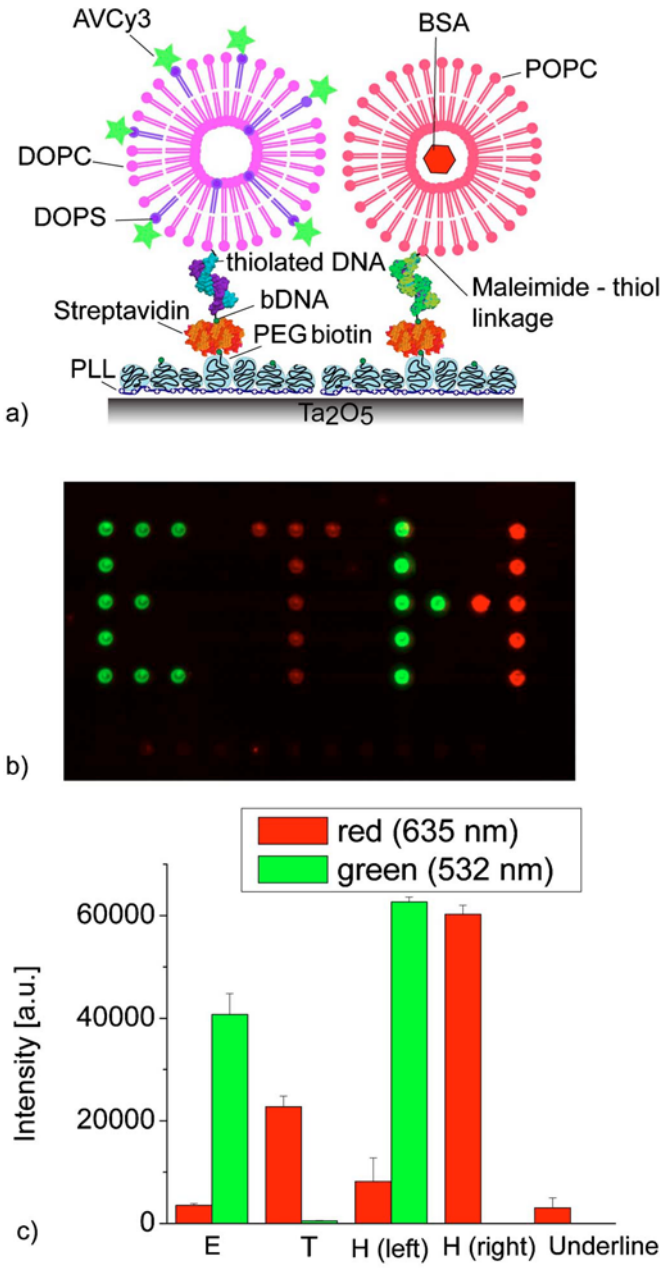

FIG. 1. (a) Schematic and (b) microarray reader image of the green and red channels of the surface immobilized vesicles. A Ta_2O_5 waveguiding chip is coated with PLL-*g*-PEG/PEGbiotin. In the second step, complexes of SA and biotinylated DNA are spotted onto the chip. Finally, the surface is exposed to a solution of two kinds of functionalized vesicles [left (E): A5Cy3 modified DOPC/DOPS vesicles; right (T): BSA647 loaded POPC vesicles] tagged with DNA via the maleimide-thiol linkage. In addition, references are included: H (left): SA532, H (right): SA633, and the underline: unlabeled streptavidin. The DNA-tagged vesicles selectively decorate the corresponding spots on the surface. (c) The mean intensity of the spots with the same modification after subtraction of the background along with the standard deviation of the fluorescent image are shown.

II. MATERIALS AND METHODS

A. Microarray chips

For all the experiments, a 160 mM buffer solution, consisting of 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (MicroSelect, Fluka Chemie GmbH, Switzerland) and 150 mM NaCl (HEPES), adjusted to *p*H=7.4 was used. The chips were cleaned by ultrasonication for 10 min in 2-propanol, rinsing with ultrapure water (MilliQ Gradient A 10 system, resistance of 18 MΩ/cm, total 4 ppb, Millipore Corporation, Switzerland), blowdrying with nitrogen, and 2 min of oxygen plasma cleaning. The surface of the Ta_2O_5 waveguiding microarray chip [Zeptosens—a division of Bayer (Schweiz) AG, Switzerland] was coated with 0.1 mg/ml biotinylated poly(L-lysine)-*grafted*-poly(ethylene glycol) (PLL-*g*-PEG/PEGbiotin) for 40 min. PLL-*g*-PEG/PEGbiotin is a graft copolymer consisting of a 20 kDa poly(L-lysine) (PLL) backbone and 2 kDa poly(ethylene glycol) (PEG) side chains grafted to it (grafting ratio =3.5) with 50% of these side chains biotin functionalized (PEGbiotin=3.4 kDa).[20] A control experiment was performed using PLL-*g*-PEG, which has the same architecture as the previously mentioned polymer but none of its side chains biotinylated. The chips were then rinsed with water and blown dry with nitrogen.

B. Spotting of complexes of streptavidin and DNA

All proteins were purchased from Invitrogen, Switzerland. All DNA sequences were purchased from Eurogentec, Belgium, and were free of self-complementarity and hairpin formation issues. For all the spotting experiments, the streptavidin/DNA solutions were diluted 1:1 vol % in ZeptoMARK spotting buffer [Zeptosens—a division of Bayer (Schweiz) AG, Switzerland]. The acronym "ETH" was chosen as the spotting template. A pin and ring spotter [GMS 417 arrayer, Affymetrix (formerly Genetic MicroSystems), USA] was used for the creation of the microarrays (ambient conditions; spot diameter ~150 μm). In order to get surfaces with spots of different functionalities, complexes of streptavidin (SA, 8 μM) and biotinylated DNA (bDNA1) (4.4 μM, bDNA1: 5′-CCC-CCA-TGG-AAT-CGT-AA-3′, 5′ modified with biotin; for all the DNA sequences, the five C bases are used as spacer) were arrayed as the "E," and complexes of SA and bDNA2 (4.4 μM, bDNA2: 5′-AAT-GCT-AAG-GTA-CCC-CC-3′, 3′ modified with biotin; the sequence is the same as bDNA1 with exchanged 3′ and 5′) were arrayed as the "T." Additionally, alexa fluor532 conjugated streptavidin (SA532, 250 μg/ml) was spotted as the left side of the "H" and streptavidin alexa fluor633 (SA633, 250 μg/ml) as the right side of the H to serve as a reference on each sample. The underline of the acronym was arrayed using unlabeled streptavidin (250 μg/ml) as a negative control. The waveguiding chip was dried for 3 h at room temperature, rinsed with water, and blown dry with nitrogen after the spotting process.

C. Vesicle preparation and functionalization

For this study, two kinds of vesicles were used. Vesicle population 1 consisted of 90 wt % 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 5 wt % 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[4-(p-maleimidophenyl) butyramide] (Mal-PE), and 5 wt % 1,2-dioleoyl-sn-glycero-

3-[phospho-L-serine] (DOPS). Vesicle population 2 consisted of 95 wt % palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 5 wt % Mal-PE. All lipids were purchased from Avanti Polar Lipids, USA. DOPC and POPC lipids are both zwitterionic, while DOPS is negatively charged. The lipids (total concentration of 5 mg/ml), dissolved in chloroform, were mixed in a round flask, and the solvent was evaporated for 1 h using nitrogen. 1 ml of HEPES was added to population 1. In order to load population 2 with a protein, 1 ml of 1 mg/ml bovine serum albumin 647 (BSA647) in HEPES was added. The vesicle solution was vortexed and extruded through 50 nm filters for 31 times. The free BSA647 was separated from the vesicles via gel chromatography (Sephadex G100, Sigma Aldrich). Both vesicle populations (500 μg/ml) were separately mixed with thiolated DNA (2 μM; tDNA1 complementary to bDNA1: 5′-CC-CCC-TTA-CGA-TTC-CAT-3′, 5′ modified with a thiol; tDNA2 complementary to bDNA2: 5′-TAC-CTT-AGC-ATT-CCC-CC-3′, 3′ modified with a thiol) in HEPES buffer for 1 h in order to tag the lipidic nanocontainers with DNA via the thiol-maleimide linkage. In the case of population 1, in addition to the thiolated DNA, 6 μg/ml Cy3 conjugated annexin A5 (A5Cy3) and 1.7 mM Ca^{2+} were added in order to functionalize the vesicles with a membrane protein which is known to specifically interact with phospho-L-serine in the presence of calcium.[21] 50 μl of each functionalized population were mixed for 10 min (with 2 μl of 10 mg/ml BSA) and then injected into the liquid cell of the microarray reader without further separation or purification. The BSA was added to suppress the nonspecific binding of the labeled BSA to the spots; it also acted to block potential errors in the PLL-*g*-PEG/PEGbiotin layer.

D. Microarray detection

A ZeptoREADER (Zeptosens—a division of Bayer (Schweiz) AG, Switzerland), a highly sensitive fluorescent microarray reader based on a waveguiding technique, was used to analyze the spotted samples. The technique makes use of the evanescent field generated by the laser light that is coupled into the waveguiding chip of the ZeptoREADER to excite fluorophores in the proximity of the surface. This way of detection, compared to conventional fluorescence scanners, is more sensitive mainly because the excitation and emission light are well separated. The device is equipped with two lasers ($\lambda = 532$ and 635 nm), the corresponding filters, and a charge-coupled device camera. More instrumental details can be found in Refs. 25 and 26. The waveguiding chip was mounted in a carrier for the microarray reader together with a liquid cell which was filled with 50 μl of HEPES. Reference images of the spotted surfaces (where only the H is detectable) were taken in order to check for the spotting quality prior to the injection of the vesicle mixture. After 48 h incubation in the dark at room temperature, images of the green and red channels were taken. For the intensity analysis of the microarray reader images, the software SENSICHIP VIEW2.1 [Zeptosens—a division of Bayer (Schweiz) AG, Switzerland] was used.

III. RESULTS AND DISCUSSION

We have designed our technique to get access to the high information content required for microarrays. Not only is the successful surface sorting of functionalized DNA-tagged vesicles onto spotted complexes of SA and bDNA shown in Fig. 1 but also some of the necessary control experiments. Figure 1(b) presents an example of the spotted acronym ETH after the surface sorting of two different kinds of vesicles; E functionalized with A5Cy3 tagged vesicles and T functionalized with BSA647 loaded vesicles. The H and the underline represent fluorescently labeled and unlabeled streptavidin, respectively. All spots were well defined without any nonspecific adsorption in the background or smearing out of the dots. Figure 1(c) shows the averaged intensity analysis of the spots of the green and red channels of the microarray reader images.

The spotted complexes of SA and bDNA maintained their activity and selectivity. The observed selectivity of the process is shown by the fact that the cross-talk between the DNA strands was negligible; only 1% green signal was detectable on T if the left side of E is set as the reference (100%). Less than 1% of the green signal was detected on the bare streptavidin spots, showing that the bDNA was necessary to immobilize the vesicles. Additionally, the exchange of the DNAs between the different kinds of vesicles, as for instance observed when the tagging is done via single cholesterol, can be avoided when using either bivalent cholesterol-based coupling[16] or the here applied maleimide-thiol linkage between the vesicles and the DNA. The stability of the vesicle-DNA linkage is indicated by the low green and red signals on T and E, respectively. Furthermore, the negatively charged vesicles (i.e., those containing DOPS) were neither attaching onto the bare streptavidin (H and underline) nor onto the PLL-*g*-PEG/PEGbiotin coated background.

The BSA647 loading of the POPC vesicles was successfully performed as shown by the large red signal on T, where the complementary bDNA strands were spotted. The red signal of E (12% if T is set as 100%) might be attributed to the nonspecific adsorption of free BSA647, which either leaked out of the vesicles or was not completely removed during the gel chromatography.

Control experiments showed that the presence of the SA was necessary in order to provide spots of single-stranded bDNA on the surface for the immobilization of the DNA-tagged vesicles. In Fig. 2(aII) column A represents spotted complexes of SA633 and bDNA1, while B contained only spotted bDNA1. The subsequent immobilization of the DNA-tagged A5Cy3 functionalized vesicles occurred only where the single-stranded DNA was immobilized via the streptavidin [Fig. 2(aII)]. In column B less than 1% of the green channel compared to column A was detectable, showing that no vesicles were surface immobilized. Although spotted complexes of SA633 and bDNA1 on PLL-*g*-PEG without the biotin function of the polymer were detectable, they were found to be much smaller and less defined [Fig. 2(bI)]. The complexes were not bound via the streptavidin-biotin linkage, but only nonspecifically dried onto the sur-

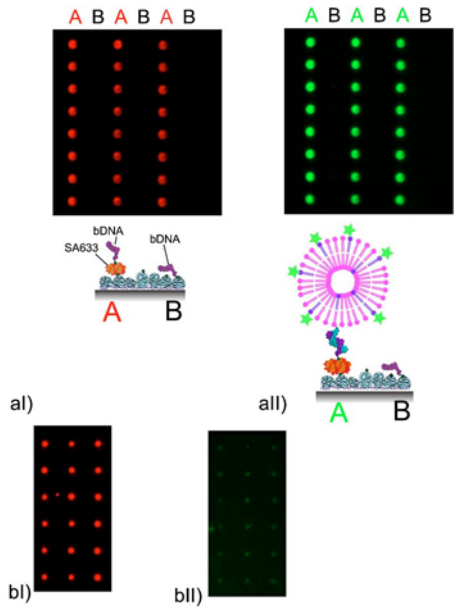

FIG. 2. (aI) Spotted array of SA633 and bDNA1 complexes (A) and bDNA1 alone (B). Subsequent immobilization of the A5Cy3 functionalized DNA-tagged vesicles occurred in column A, while in column B a green signal which is less than 1% compared to column A is detected (aII). Spotting onto PLL-*g*-PEG without the biotin function leads to smaller spots of SA633 and bDNA1 (bI), and only 2% of vesicles compared to (aII) column A can be surface immobilized (bII).

face. In addition, only 2% of A5Cy3 functionalized vesicles tagged with the complementary DNA were immobilized onto such spots compared to Fig. 2(aII) column A [Fig. 2(bII)]. There was no observable affinity difference for the vesicle hybridization depending on the use of SA or SA633 as shown in Fig. 2(aII), where the vesicle immobilization is similar to that in Fig. 1(b), letter E. This observation indicates that the complexes of SA633 and bDNA1 were dried between the nonfunctionalized PEG chains and therefore not accessible for the vesicles tagged with the complementary DNA.

IV. CONCLUSION

We have shown that protein loaded or membrane-binding protein functionalized vesicles can be arrayed via surface sorting using DNA as a linker. Different types of vesicles were successfully guided onto the corresponding predefined spots containing the complementary DNA strand. It was possible to immobilize vesicles loaded with BSA647 and vesicles functionalized with the membrane-binding protein A5Cy3 via surface sorting. The background was resistant towards the nonspecific adsorption of the vesicles and their protein functionality. As a next step, the approach could be extended to other transmembrane proteins such as ion channels or G-protein coupled receptors.

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