Lipoconjugates for the Noncovalent Generation of Microarrays in Biochemical and Cellular Assays

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The generation of microarrays by functionalization of hydrophobic glass surfaces with conjugates of triacylated lipophilic end-groups and with a peptide or hapten as a test substance is presented. Immobilization on the hydrophobic surfaces through the triacylated anchor group is fully orthogonal to the reactivity of functional groups within the test substances. The technique is therefore free of risk that reactions of these functional groups may influence the biological activity of the test compounds in screening applications. In addition, no preactivation of either the surface or the compounds is required. Reagents and substrates may be stored at ambient conditions for long periods of time. The lipoconjugates are administered from aqueous solution enabling automated nanopipetting down to spot dimensions of 100 μm across. The microstructures are stable with respect to the conditions of biochemical assays and applications in cell biology. Due to the hydrophobicity of the nonfunctionalized surfaces, standard blocking protocols used in microtiter-plate testing can be employed, thereby inhibiting nonspecific binding of assay reagents. Generation of these microstructures on hydrophobic glass slides or coverslips enables highly sensitive multichannel read-outs with high-resolution fluorescence microscopy.

KEYWORDS:

bioassays · fluorescence microscopy · lipids · microarrays · surface chemistry

Introduction

The microstructured immobilization of test compounds on surfaces in the form of microarrays has opened up new dimensions in terms of parallelization in high-throughput screening and diagnostics.[1–4] While most of the applications today involve oligonucleotide-based arrays, used for example for expression screening or identification of gene mutations in cancer diagnostics,[5, 2] peptide arrays in diagnostics[6] and arrays of drug-like compounds in drug-screening applications are gaining significance.[7, 3]

For the generation of microarrays, two strategies can be discriminated. In the first case, the substances are synthesized on the microarrays, either by photolithographic techniques involving photolabile protecting groups[8] or by synthesis procedures used in solid-phase chemistry.[9] Synthesis starts with an anchor group covalently attached to the support to which subsequent building blocks are coupled in a chemically defined way. At the end of the synthesis, the protecting groups are removed.[9] However, in this strategy, side-reactions and incomplete turnover may lead to a heterogeneous mixture of molecules with misleading assay results. In addition, synthesis protocols need to be compatible with the array format and chemical properties of the surface.

In the second case, the compound collection is immobilized on the microarray by spotting or nanopipetting devices[3] *postsynthesis*. Substances can be fully characterized analytically prior to spotting, and a multitude of arrays can be produced from one batch of substance. Spots of 150 – 200 μm in diameter still allow up to 1600 testings per square centimeter.

Immobilization of molecules takes place either through strong noncovalent interactions, as for large DNA molecules, or by covalent linkage through functional groups. In most cases, such functionalities are nucleophilic groups of the compounds reacting with surface reactive groups, such as epoxy groups, succinimidylesters, or maleimido groups. Following this strategy, two problems are commonly encountered. The first is that the surface reactive groups are sensitive to water. Once exposed to ambient conditions, a preactivated surface needs to be processed quickly. Moreover, water is the preferred solvent in nanopipetting applications due to its superior characteristics in drop generation, as well as spot diameter when compared with

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organic solvents. Unless immobilization occurs through functional groups with a much higher reactivity than water, quenching of reactive groups by water will limit the efficiency of substance immobilization.

The second problem encountered is the difficulty in achieving full directionality of surface functionalization by one specific functional group within the compound. For example, if immobilization occurs through amino groups, any amino group within a compound may react with the surface, thereby leading to a heterogenous mixture of immobilized compounds. Similarly, even though sulfhydryl groups exhibit a higher reactivity for a Michael addition on maleimides, amino groups may still engage in this reaction. Recently, Hergenrother et al. presented a strategy in which the reaction of primary alcohols was strongly preferred over the reaction of secondary, phenolic, and methyl ether alcohols.[7] However, even if such orthogonality is achieved, specific immobilization through any type of nucleophilic group imposes a severe restraint on the diversity of a combinatorial compound collection with respect to the medicinal-chemistry aspects of compound libraries. Nucleophilic groups are highly relevant for interactions of potential drugs with target molecules.

To overcome the above-mentioned limitations, immobilization strategies are required that are either fully orthogonal to any functionality relevant with respect to the medicinal chemistry of compound collections or afford a site-directed surface functionalization. In addition, array substrates and compounds should be insensitive to water and stable at ambient conditions. Finally, the strategy should be compatible with nanopipetting devices.

Two strategies enabling a site-directed covalent coupling for the generation of microarrays have been presented so far. Stolowitz et al.[10] introduced the complexation of phenylboronic acid with salicylhydroxamic acid modified Sepharose as a chemoselective linker. This complexation strategy may be transferred to microarray substrates. The formation of the complex is pH-dependent. Immobilized compounds can be released from the substrate by a decrease in the pH value. Falsey et al.[11] exploited the formation of thiazolidines or hydrazones from a glyoxyl functionality and either oxyamines or 1,2-aminothiols for surface functionalization. The glyoxyl group is immobilized on amino-functionalized glass substrates. In order to obtain small spots, the glyoxyl-modified surface has partially hydrophobic properties. This strategy has been applied to the generation of microarrays for the screening of small molecules binding to fluorescently labeled probe molecules and for the identification of molecules mediating cell adhesion in cellular microarray applications.

These site-directed immobilization strategies, like those involving reactive groups in general, rely on the formation of covalent bonds. We reasoned that, for small molecules, strong hydrophobic interactions mediated by an anchor group common to all molecules could provide a further avenue to site-directed immobilization, similarly to the immobilization of DNA molecules through strong ionic interactions.

The triacylated lipid *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine (Pam_3Cys)[12] and the structurally related tripalmitoylated *S*-(1,2-dicarboxyhexadecyl)ethyl-*N*-palmitoyl-L-cysteine (PHC)[13] had been employed previously for the immobilization of peptides, which were conjugated to either anchor group, on the plastic surface of wells in microtiter plates. The peptide moieties of such constructs were still specifically recognized by antibodies in ELISA-type experiments;[14] apparently, the Pam_3Cys anchor group did not impair the accessibility of the peptide. Considering the suitability of these lipoconjugates in the microtiter-plate experiments, we decided to determine whether the lipophilic functionalities could also serve as anchor groups for the generation of microarrays in drug-screening applications with binding proteins. Glass supports rendered hydrophobic by silanization with octadecyltrimethoxysilane served as the substrate.

The lipophilic anchor group can be readily incorporated into combinatorial compound collections generated by automated solid-phase chemistry. Resins preloaded with the triacylated Pam_3Cys building block have been established, and work-up procedures and HPLC analytical protocols have been optimized (F. Reuter, K.-H. Wiesmüller, G. Jung, unpublished data).

Test compounds carrying either a peptide functional group or biotin as a hapten were synthesized on Pam_3Cys- or PHC-preloaded resins. To assess the molecular state of these substances in solution and to monitor surface functionalization in time-lapse experiments, fluorescein-labeled derivatives were generated. In spite of the lipophilic anchor groups, all compounds were readily dissolved in a two-step procedure with tertiary butyl alcohol as the initial solvent followed by dilution into double-distilled water (ddH_2O). Samples were pipetted, either manually or by using a nanopipetting device, from aqueous solutions and dried in ambient conditions. The peptide and the hapten functional groups were detected specifically by using fluorescent antibodies and fluorescently labeled streptavidin.

In contrast to protocols employing acylated compounds in the formation of solid-supported lipid bilayers,[15] in our case, the substrates may be dried and can be stored in a dried form for long periods of time. Even though the generation of microstructured solid-supported bilayers has been demonstrated,[16] the need to permanently maintain aqueous conditions imposes a severe limitation on the handling and storage of such functionalized surfaces. While solid-supported bilayers possess membrane fluidity and are therefore superior in mimicking the environment of biological membranes, membrane fluidity is not a prerequisite in screens of molecular interactions.

In addition to demonstrating the potential of this novel functionalization strategy for the generation of microarrays in binding experiments with isolated proteins, cellular applications were explored as well. Lipoconjugates carrying a biotin headgroup were spotted onto hydrophobic glass coverslips, and then incubated with streptavidin and a biotinylated antibody directed towards cell-surface molecules. Specific interaction with cell-surface molecules of suspended cells was detected by the induction of calcium signals in Jurkat T cell lymphoma cells and retention of cells expressing cell-surface markers on the functionalized surfaces. The lipoconjugate-based surface functionalization is stable to the conditions of tissue culture over

several days, thereby allowing cell growth of adherent tissue culture cells on these microstructured surfaces.

Results

Conjugates of the lipophilic Pam_3Cys or PHC anchor groups and peptides, as well as biotin, were synthesized by automated solid-phase synthesis on Pam_3Cys- or PHC-preloaded resins (Scheme 1). Cleavage of the reaction products from the resin and the workup followed protocols commonly employed for peptides.

Comparison of solvents for the generation of homogeneously functionalized spots

Optimum conditions for the generation of homogeneously functionalized and regularly shaped spots were identified by dissolving a fluorescently labeled peptide lipoconjugate into a series of organic solvents and water and pipetting one microliter of the test solutions manually onto glass coverslips rendered hydrophobic by silanization with octadecyltrimethoxysilane. After drying, the distribution of fluorescence was analyzed by confocal fluorescence microscopy (Figure 1).

To dilute lipoconjugates into water, the substances were initially dissolved in tertiary butyl alcohol/water (4:1) followed by dilution into water to the desired concentration. Volatile apolar organic solvents were included to address whether solvent conditions matching the apolarity of the acyl side chains promoted the homogeneity of the surface functionalization. To assess the degree to which the rate of solvent evaporation affected the surface functionalization, *N*,*N*-dimethylformamide (DMF) was included as a less-volatile polar organic solvent.

Volatile organic solvents, such as dichloromethane or toluene, resulted in highly irregular structures and a poor transfer of lipoconjugate from the pipette onto the coverslip because of rapid evaporation of the solution at the pipette tip (Figure 1 A). Less-volatile organic solvents, such as DMF, spread rapidly on the silanized glass and yielded large, irregularly functionalized areas (Figure 1 B). In contrast to the organic solvents, well-defined round spots and a homogeneous functionalization were obtained with aqueous solutions (Figure 1 C). Prolonged storage of drops in atmospheres vapour-saturated with the respective solvent did not improve the surface functionalization.

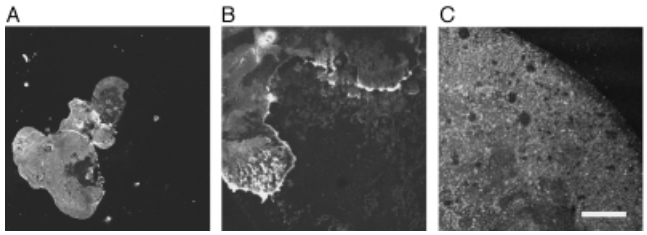
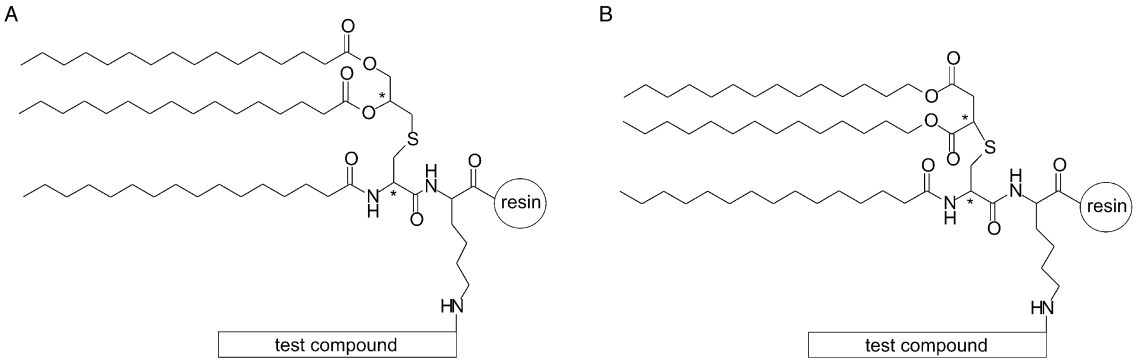


Figure 1. *Comparison of solvents for the immobilization of a fluorescein-labeled peptide lipoconjugate on an octadecyltrimethoxysilane-functionalized glass coverslip. Examples are shown for organic solvents of high and low volatility: A) toluene, B) DMF, and C) water. Highly volatile solvents such as toluene resulted in poor transfer of compound from the pipette tip to the substrate and irregular spot shapes and diameters, whereas organic solvents with low volatility such as DMF resulted in uncontrolled spread of the solution. Aqueous solutions yielded regular round spot shapes and homogeneity in surface modification. The bar denotes 200 μm.*

Process of surface functionalization

After aqueous solutions were identified as the optimum condition for pipetting, fluorescence at the surface of the coverslip during evaporation of solvent was monitored by time-lapse confocal microscopy (Figure 2). These analyses of the process of functionalization served to provide a basis for a rational optimization of the immobilization protocol. Confocal microscopy was employed to detect only fluorescence originating from molecules bound to the surface and close to the surface.

The fluorescent lipoconjugate was enriched on the surface of the drop, which can be explained by the tendency of the hydrophobic end groups to orient themselves at the air – water interface. After drop deposition, an increase of surface fluorescence was observed (1 – 3 min). Only a small transfer of lipoconjugate from the drop surface onto the coverslip was observed during retraction of the drop surface during the drying process. Lipoconjugate accumulated at the drop surface and then was deposited at the position at which the drop finally dried. The sudden decrease in fluorescence is explained by self-quenching of fluorescein at high concentrations and by the solvation dependence of fluorescein fluorescence. The size and



Scheme 1. *Molecular structures of the lipophilic Pam_3Cys (A) and PHC (B) anchor group attached to the resin for combinatorial solid-phase synthesis of lipoconjugate-anchored compounds. Spacer groups may be introduced between the anchor group and the test compound.*

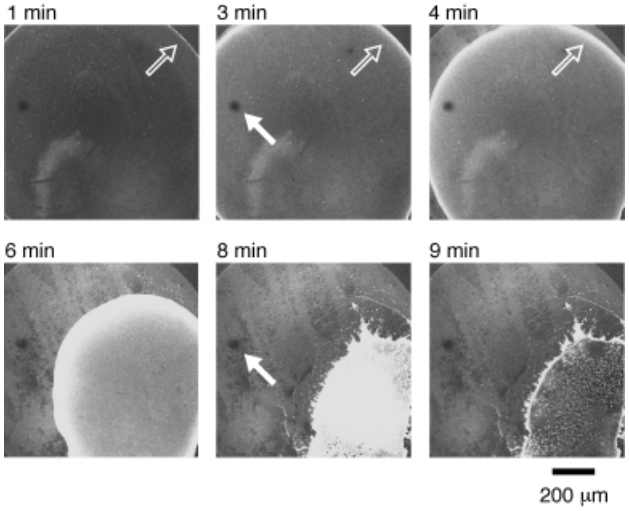


Figure 2. *Process of surface functionalization during drop evaporation. Aqueous solution (1 μL) of a fluorescein-labeled Pam_3Cys – peptide conjugate was pipetted manually onto a hydrophobic glass coverslip. The drying process was followed by confocal laser scanning microscopy focused on the surface of the coverslip. The time elapsed between pipetting and image acquisition is indicated for each picture. Only fluorescence from molecules at the surface or in the solution close to the surface is detected because of the confocal detection. The open arrows at times of 1 – 4 min point to the retracting drop surface. While the fluorescence on the surface of the coverslip remains constant, the fluorescence on the surface of the drop increases with the reduction in drop volume. The sudden decrease of fluorescence intensity after drying (t = 9 min) is explained by the solvation dependence of fluorescein fluorescence and self-quenching at high densities. The presence of the dark spot (closed arrow), probably the result of a defect in surface silanization, throughout all images illustrates that surface functionalization largely occurred during the initial contact of the drop with the surface.*

shape of the spot represented the contact area of the drop with the glass surfaces. The observation that only a little transfer of lipoconjugate occurred during retraction of the drop is indicative of a surface functionalization primarily during the initial contact of the drop with the hydrophobic surface. Consistent with this process of surface functionalization, prolonged incubation of drops on surfaces in vapour environments saturated with the appropriate solvent did not increase the homogeneity of the functionalization.

Structural analysis of lipoconjugates in solution and on surfaces

Previously, surface functionalization with compounds having lipid end groups was intended to yield intact lipid layers on a hydrophobic support. Such solid-supported lipid bilayers are characterized by the presence of lateral mobility of probe molecules incorporated into the lipid layer[17] and by a smooth surface structure.[18, 19] For the generation of such bilayers, the lipids are transferred to the surface either by vesicular fusion of small unilamellar vesicles (vesicle spreading) or by monolayer transfer.[15] Throughout the process and thereafter, surface hydration has to be maintained. Prass et al. have presented the formation of monolayers of the Pam_3Cys anchor group with a film balance.[20] In contrast to these protocols, in our case the spots were pipetted from solutions of lipid conjugates and allowed to dry. In order to compare the structure of the lipoconjugates deposited on the surface by nanopipetting with that of solid-supported bilayers, our data obtained by confocal laser scanning microscopy were complemented by surface analyses with atomic force microscopy (AFM)[21], as well as by analyses of the molecular state of the lipoconjugates in solution.

Spots pipetted from aqueous solutions yielded sharp edges and good overall homogeneity. Some heterogeneity of surface fluorescence was observed on the micron scale (Figure 1C). It had been described previously, that Pam_3Cys conjugates form vesicular or tubular aggregates of different sizes in aqueous solutions depending on the configuration of the glycerol moiety and the head group.[22] The basis for the microheterogeneity of the functionalized surfaces was addressed by analyzing aggregate formation of lipoconjugates in solution. Fluorescence correlation spectroscopy (FCS) was employed for this purpose.[23, 24] FCS derives information on molecule numbers, as well as molecule size, from the analysis of temporal fluctuations emanating from fluorescently labeled molecules passing through a femtoliter detection volume, defined by confocal detection optics. Atomic force microscopy served to study the molecular state of a fluorescein-labeled peptide lipoconjugate on the surface. FCS of aqueous solutions revealed that the lipoconjugate is in fact present as a heterogeneous population of aggregates (Figure 3A). With a diffusion constant about 50 – 500 times smaller than that for a free variant of green fluorescent protein (GFP), a barrel-shaped protein with dimensions of about 3×4 nm, the aggregate size for the lipopeptide conjugates is in the order of 200 nm – 2 μm. These dimensions were confirmed by atomic force microscopy of lipoconjugates on dried surfaces (Figure 3B). Further studies will be needed to elucidate the molecular structure after rehydration.

Lipoconjugates for the detection of ligand – receptor interactions

After having analyzed the process of surface functionalization and the structure of the lipoconjugate on the surface, the conditions for surface functionalization were validated for further nonfluorescent peptide lipoconjugates, as well as for a Pam_3Cys – biotin conjugate. As these molecules were not fluorescent by themselves but required binding of fluorescently labeled probe molecules for visualization, these experiments also served to explore the use of lipoconjugates in microarray-type protein binding assays.

Surface functionalization was carried out at room temperature followed by further drying at 37 °C for 1 h. Room temperature was identified as the optimum temperature for surface functionalization. In order to probe for binding of lipoconjugates with specific probe molecules, the coverslips were incubated with fluorescently labeled streptavidin and an α-Myc-tag antibody followed by a fluorescently labeled secondary antibody (see Experimental Section). In order to avoid unspecific binding, the surfaces were blocked with buffers containing bovine serum

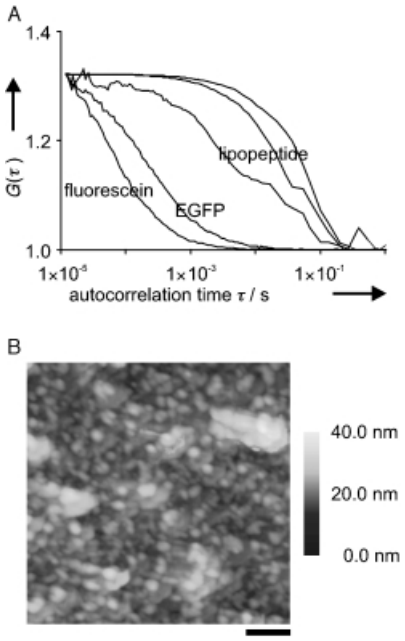


Figure 3. *Structural characterization of a fluorescein-labeled peptide Pam_3Cys lipoconjugate A) in aqueous solution and B) on a hydrophobic glass surface. A) Fluorescence correlation spectroscopy. Reference measurements were carried out for free fluorescein and the EGFP variant of the green fluorescent protein (GFP). In contrast to the reference molecules, autocorrelations with long diffusional autocorrelation times were obtained that greatly differed for a number of sequential measurements, indicative of a heterogeneous population of aggregates with different sizes. B) AFM of a Pam_3Cys – peptide conjugate deposited on a hydrophobic glass surface from aqueous solution at a concentration of 9 μM. AFM measurements were performed in the dried state as described in the Experimental Section.*

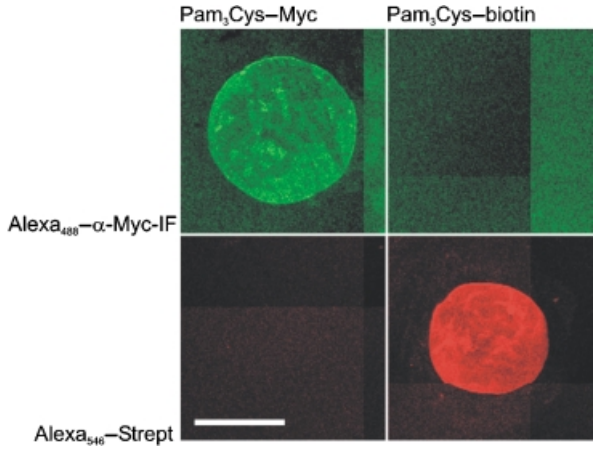


Figure 4. *Lipoconjugates in parallel biochemical binding assays. The PHC – biotin lipoconjugate and a peptide with the Myc-tag sequence conjugated with Pam_3Cys through its N terminus were manually spotted onto two distinct sites of a silanized coverslip. The hapten head-group was specifically detected by $Alexa_{546}$-labeled streptavidin, and the Myc tag was detected by indirect immunfluorescence with an α-Myc-tag monoclonal antibody and an $Alexa_{488}$-labeled antimurine secondary antibody. The bar denotes 500 μm. The images were acquired by confocal laser scanning microscopy on a whole 12 mm diameter coverslip in a mosaic-like fashion. The different shading of the rectangles in the images results from slight differences in the brightness of each frame.*

albumin (BSA) prior to incubation with the probe molecules. The peptide lipoconjugate, as well as the biotin, were recognized specifically by their respective probe molecules (Figure 4). By combining of $Alexa_{546}$-labeled streptavidin with an $Alexa_{488}$-labeled secondary antibody, both the biotin lipoconjugate and the Myc-tag lipoconjugate could be detected simultaneously on one single coverslip. No unspecific binding of either probe molecule was present. Clearly, the probe molecules bind to the functional head-group and not to the lipid anchor. These results establish lipoconjugates as a functionalization strategy for the parallel identification of ligand – target interactions with small-molecule microarrays.

Homogeneous surface functionalization was achieved at concentrations ranging from 4 – 50 $\mu g\,mL^{-1}$. Higher concentrations led to an accumulated deposition of conjugate at the end of the evaporation process; this was washed off when aqueous buffer was added in biochemical assays. At lower concentrations, the density of lipoconjugates decreased and sometimes a segmented appearance of the spots was obtained.

Lipoconjugates in the generation of small-molecule microarrays

Manual pipetting of microliter volumes yielded spots with diameters of about 1 mm. For the generation of protein, peptide, and small-molecule microarrays, automated nanopipetting devices are employed, enabling pipetting volumes of less than 1 nL to be used and resulting in spot diameters of about 150 microns. At a spot-to-spot lateral distance of 250 microns, 1600 interactions can be probed conveniently on a single square centimeter.

Pipetting such small volumes differs from pipetting microliter volumes in that spots dry nearly immediately at ambient conditions; this strongly affects the time available for molecules to react with the surface of the substrate. For this reason, the performance of the lipoconjugates for the generation of small-molecule microarrays was assessed by using an automated, piezoelectric nanopipetting device. Aqueous solutions of the Pam_3Cys – biotin conjugate, as well as of the Pam_3Cys – Myc conjugate, were pipetted in an array format onto a silanized glass coverslip. Piezoelectric pipettors may pipette a multiple of a minimum sample volume. In this case, multiples of the 0.5 nL pipette-specific minimum sample volume were dispensed. In the same way, for the manually pipetted lipoconjugates good homogeneity was obtained in all cases for concentrations of 3 – 30 μM (Figure 5 A, B).

To compare the performance of the lipoconjugate-based surface functionalization with established techniques, a succinimidyl ester activated biotin was pipetted onto an amino-functionalized glass coverslip (Figure 5 C, 6). A concentration of 20 μM was employed. The homogeneity of surface functionalization was comparable for both immobilization strategies. Owing to the hydrophobicity of the hydrophobic glass substrate, slightly smaller spots were obtained. Both types of biotin conjugate were readily detected with a fluorescently labeled streptavidin as a probe molecule.

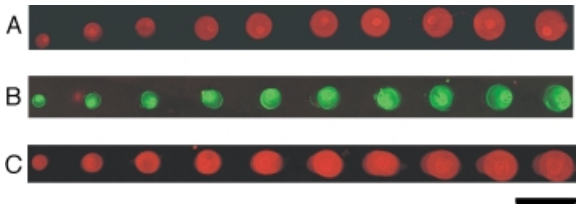


Figure 5. Generation of microarrays with lipoconjugates and compounds preactivated with a succinimidyl ester. The Pam_3Cys – biotin conjugate (A), the Pam_3Cys – Myc-tag conjugate (B), and a biotin succinimidyl ester (C) were pipetted onto a hydrophobic coverslip (A, B) or an aminosilanized surface (C) in volumes of 0.5 – 5 nl in steps of 0.5 nL. The presence of biotin was probed by an $Alexa_{546}$-conjugated streptavidin, the Myc tag was detected by indirect immunofluorescence with an $Alexa_{488}$-conjugated secondary antibody.

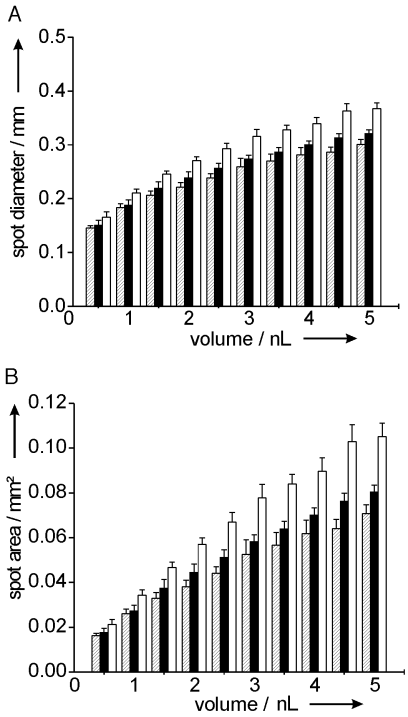


Figure 6. Dependence of spot diameter and spot area on pipetted volume for lipoconjugates on hydrophobic coverslips and the biotin-succinimidyl ester on amino-functionalized slides. Substances were pipetted onto hydrophobic coverslips or amino-functionalized coverslips as shown in Figure 5. Each bar represents a mean of ten spots pipetted with the same volume. Hatched bars: Myc-tag lipoconjugate on hydrophobic coverslip; closed bars: biotin lipoconjugate on hydrophobic coverslip; open bars: biotin succinimidyl ester conjugate on amino-functionalized coverslips.

Lipoconjugates in cell-based microarrays

The stability of the lipoconjugate functionalization in in vitro protein binding assays prompted us to test whether this surface functionalization could also be employed in cellular microarrays. For these cellular applications, the PHC anchor group was employed instead of the Pam_3Cys anchor group. In cell biology, molecules containing the Pam_3Cys moiety are known to act as mitogens, that is, substances that stimulate the proliferation of certain immune cells[25] through stimulation of Toll-like receptor 2 (TLR2) molecules.[26] In contrast to this, PHC is inactive with respect to TLR activation (own unpublished results).

Adherent tissue culture cells expressing a fusion protein of the human MHC class II molecule HLA-DRB*0101[27] with the GFP[28] were grown for one day on hydrophobic coverslips, locally functionalized with a biotin lipoconjugate followed by BSA blocking and incubation with fluorescently labeled streptavidin. The GFP reporter group enabled the simultaneous detection of the surface functionalization, as well as of the cells by fluorescence microscopy, without the need for immunofluorescence staining. The functionalization was maintained with sharp edges over more than a day (Figure 7 A). Cell spreading and growth was comparable for streptavidin-functionalized and -nonfunctionalized BSA-blocked surfaces. The homogeneity of the surface functionalized with PHC – biotin was comparable to that of those functionalized with Pam_3Cys – biotin.

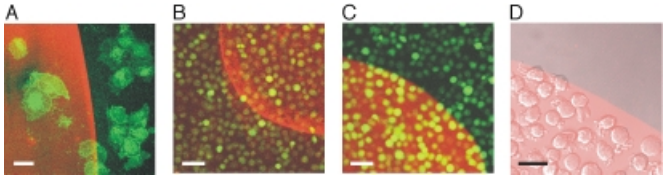


Figure 7. Growth, stimulation of cellular signal transduction, and surface-marker-dependent retention of cells on surfaces locally functionalized with lipoconjugates. A) Growth of adherent cells expressing a fusion protein of the MHC class II molecule HLA-DR1 and GFP, on a glass coverslip locally functionalized with fluorescently labeled streptavidin (red: $Alexa_{546}$-streptavidin; green: GFP-fusion protein). B, C) Stimulation of Ca^{2+} signaling in Jurkat T cell lymphoma cells. C) A streptavidin-functionalized coverslip as used in (A) was incubated with a biotinylated anti-CD 3ε antibody. The Ca^{2+}-sensitive fluorophore Fluo-3 was employed to monitor the increase in intracellular calcium by confocal laser scanning microscopy. Cells were added to the medium and allowed to settle onto the surface by gravity. B) Antibody-free control. D) Specific retention of Jurkat T cell lymphoma cells expressing the CD3 cell-surface antigen by surfaces functionalized with the anti-CD3ε antibody. Cells settled on the surface that were not functionalized with anti-CD3 antibody were washed away. The functionalization is visualized by means of $Alexa_{546}$-labeled streptavidin. In (A) and (D), the bars denote 20 μm, in (B) and (C) 50 μm.

Next, we tested whether the surface functionalization with the biotin lipoconjugate could be used for the immobilization of biomolecules interacting with cell-surface proteins and eliciting a specific cellular response. Such assay formats are valuable tools for the identification of antibodies, antibody-like molecules, and other molecules specifically interacting with cell-surface molecules. Human Jurkat T cell lymphoma cells, loaded with the calcium-sensitive fluorophore Fluo-3, were added to coverslips locally functionalized with a biotinylated anti-CD3ε antibody. Engagement of the CD3 complex by specific antibodies is known to elicit a calcium signal in Jurkat cells.[29] As soon as cells contacted the antibody-functionalized surface, an increase in intracellular calcium levels was observed (Figure 7 B). In contrast to this, no calcium signals were observed for cells contacting the surface next to the antibody-functionalized spots or on surfaces functionalized with streptavidin only (Figure 7 C). After washing the functionalized coverslips, Jurkat cells remained only at those places where antibody was present (Figure 7 D). These results confirm that the surface functionalization is compatible with specific probing of molecules with respect to their activity in functional cellular assays as well as their interaction with cell-surface molecules.

Discussion

The immobilization of lipoconjugates represents a novel and versatile approach to the generation of small-molecule microarrays and surface functionalization in cell-based microarrays. The lipophilic moiety represents an anchor group that has not been utilized in this manner before. The surface functionalization is based on hydrophobic noncovalent interactions only, thereby eliminating problems arising from the insufficient long-term stability and from the orthogonality of immobilization protocols involving the reaction of preactivated compounds or surfaces. Rapid evaporation of the water does not compromise the immobilization of compounds.

The surface functionalization was fully explained by time-lapse confocal fluorescence microscopy, which demonstrated the analytical potential of fluorescence microscopy in conjunction with fluorescent conjugates for process control and optimization in the generation of microstructures. Fluorescence correlation spectroscopy, as well as atomic force microscopy, revealed that the lipoconjugates were present as a heterogeneous population of aggregates of different sizes. Experiments in which the fluorescence was locally photobleached (fluorescence recovery after photobleaching (FRAP) technique)[30] revealed that no lateral diffusion occurred within the functionalized areas (not shown), in contrast to solid-supported bilayers.[17] This observation, again, is consistent with an aggregated, micellar state of the lipoconjugates, in which no continuous monolayer is formed upon deposition on the hydrophobic support. Prolonged incubation of the aqueous solutions had no effect on the surface functionalization.

The lipoconjugates are fully compatible with dispensing by piezoelectric nanopipettors for the generation of low-density microarrays. Due to the hydrophobicity of the substrate, smaller spot diameters were obtained for the lipoconjugates than for an aqueous solution of a biotin succinimidyl ester pipetted onto an aminosilane-functionalized glass coverslip. This difference was more pronounced for larger volumes.

Both biotin lipoconjugates were readily detectable by fluorescently labeled streptavidin. Evidently, the slightly larger size of the Pam_3Cys anchor group is not a limiting factor for surface functionalization compared to the immobilization by smaller succinimidyl ester groups. A molecular density of about 0.5 molecules per nm^2 was obtained for monolayers of Pam_3Cys conjugates.[20] Amino functionalization was reported to yield a maximum of two amino groups per nm^2.[31] However, in both cases, for recognition by proteins, the size of the protein rather than differences in the density of surface functionalization is the limiting factor for the maximum signal obtained by protein binding. For the lipoconjugates, a homogenous surface functionalization was obtained over a concentration range of 3 – 30 μM. For a pipetted volume of 0.5 nL and a spot area of 0.018 mm^2, immobilization of all molecules from a 20 μM solution results in a spot density of 0.3 molecules per nm^2, which is close to the value given for monolayers in ref. [20].

Small-molecule compound collections for the generation of such microarrays can be readily generated by automated solid-phase chemistry on resins preloaded with the anchor group. In this report, this strategy was exemplified for the Myc-tag and biotin lipoconjugates. Due to the chemical stability of the Pam_3Cys moiety and the absence of reactive groups, many protocols of solid-phase combinatorial organic synthesis can be readily transferred onto this novel type of resin. High-throughput analytical HPLC coupled with ESI-MS and purification of Pam_3Cys-derivatized peptides have been established (F. Reuter, K.-H. Wiesmüller, G. Jung, unpublished data). Alternatively, the Pam_3Cys building block may be coupled postsynthesis onto an available amino group.

Based on the Pam_3Cys – biotin as well as the PHC – biotin conjugates, a versatile streptavidin matrix for surface functionalization was assembled on a coverslip and employed in cellular microarray applications to analyze the interaction of probe molecules with cell-surface molecules. This multilayer assembly allowed noncovalent immobilization of recognition molecules with full preservation of specificity and function, and the use of this surface for the discrimination of cell-surface antigens. The noncovalent functionalization provided sufficient strength for mediating interactions with cells through the recognition molecules and proved to be stable in cell culture for at least two days. To avoid activation of signal transduction in cells expressing Toll-like receptor 2 molecules,[32] the PHC – biotin molecule may be the preferred compound for this type of application.

A number of publications have exploited the functionalization of surfaces by using chemically preactivated biotin as an anchor group for the immobilization of streptavidin followed by pipetting biotinylated screening compounds.[33, 34, 35] In addition, the incorporation of lipids with biotin head-groups into solid-supported bilayers has been described.[36, 15] As mentioned before, our strategy overcomes the limitations in reagent and substrate stability inherent to the use of preactivated compounds or the formation and maintenance of lipid bilayers.

The lipoconjugates add a further strategy to the still very limited set of protocols for a fully orthogonal, site-directed surface immobilization of molecules for the generation of peptide and small-molecule microarrays. In contrast to the other site-directed strategies, the lipoconjugate-based approach relies on noncovalent interactions. While large DNA molecules are routinely immobilized by strong noncovalent ionic interactions with amino-functionalized cationic substrates, a comparable strategy has been missing for small molecules. The lipid anchor introduces a moiety with a physicochemical characteristic common to all probe molecules, chemically different but functionally similar to the phosphate backbone of the DNA. More detailed comparisons of the site-directed techniques with respect to process stability and control of surface functionalization will have to be carried out to assess the strengths and weaknesses of the individual approaches. Due to the high contact angles of aqueous solutions on hydrophobic surfaces, the lipoconjugate-based strategy yields spot sizes smaller than those obtained on more hydrophilic substrates, a fact enabling a further increase in sample density on pipetted microarrays.

Experimental Section

Silanization of glass coverslips: For the immobilization of lipoconjugates, the surface of type I glass coverslips was rendered hydrophobic by silanization with octadecylacyl chains. Activation of surface hydroxy groups and cleaning of the glass surface before functionalization was achieved by treating the coverslips with 1 N NaOH for 2 h, followed by washing with ddH_2O, drying in an air stream, and incubation in piranha solution (concentrated H_2SO_4/30% hydrogen peroxide (2:3)) for 45 min. The coverslips were thoroughly rinsed in ddH_2O and carefully dried with a lint-free paper tissue. For silanization, the coverslips were submersed in octadecyltrimethoxysilane (Sigma-Aldrich, Seelze, Germany) at room temperature for 18 h and then repeatedly washed in toluene and ddH_2O, until the surfaces were perfectly clean.

Amino functionalization of coverslips: Amino functionalization of coverslips followed a protocol similar to the one described in refs. [3, 31] Type I glass coverslips of 12 mm diameter were cleaned with acetone (Acetone Analytical Reagent, Riedel-de Haen, Seelze, Germany) for 15 min in an ultrasound bath and dried, then the surface hydroxy groups were activated by incubation in piranha solution for 15 min in an ultrasound bath. The coverslips were rinsed thoroughly in ddH_2O and dried in an air stream; this was followed by treatment with 3-aminopropyltriethoxysilane (3% (v/v); ABCR GmbH & Co. KG, Mannheim, Germany) in 95% ethanol (v/v with water) for 1 h. The silane solution was mixed in an ultrasound bath for 5 min before treating the coverslips to allow for hydrolysis and silanol formation. The coverslips were washed briefly with 99% ethanol, thermally cured at 100 °C for 1 h, washed again with 99% ethanol, dried in an air stream, and stored under argon until use.

Synthesis of lipoconjugates of *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine and *S*-(1,2-dicarboxyhexadecyl)-ethyl-*N*-palmitoyl-L-cysteine: The peptide moiety of the conjugates was synthesized by solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an automated peptide synthesizer for multiple-peptide synthesis (RSP5032, Tecan, Hombrechtlikon, Switzerland). Fmoc-protected amino acids were purchased from Novabiochem (Heidelberg, Germany), the spacer building block Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-Ado) came from Neosystem (Strasbourg, France). Standard chemicals in peptide chemistry were obtained from Fluka (Deisenhofen, Germany) and Merck (Darmstadt, Germany), solvents were p.a. grade. A PHC – biotin conjugate, a Pam_3Cys – biotin conjugate and a resin preloaded with Pam_3Cys-Ser-Lys for synthesis of peptides with a C-terminal lipid anchor were obtained from EMC microcollections GmbH (Tübingen, Germany). The identity of all lipoconjugates was confirmed by ESI or MALDI-TOF mass spectrometry. The amino acid sequence of the α-Myc tag was EQKLISEEDL; two Ado building blocks were inserted between the Myc tag and the Pam_3Cys lipid anchor, either at the N or the C terminus.

Synthesis of the lipoconjugate with N-terminal Pam_3Cys proceeded by first synthesizing the peptide moiety on a Rink-amide resin (Novabiochem, Darmstadt Germany), followed by coupling of a fivefold excess of Fmoc-Ado. The coupling reaction was carried out in the presence of five equivalents each of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and (*N*-hydroxybenzotriazole (HOBt) and ten equivalents of diisopropylethylamine (DIPEA) in DMF for 16 h. 5,6-Carboxyfluorescein was coupled in the same way. A fivefold excess of the Pam_3Cys lipid anchor dissolved in dichloromethane was coupled in the presence of five equivalents of HOBt/*N*,*N*′-diisopropylcarbodiimide (DIC). Prior to the N-terminal modification, the identity and purity of the product was confirmed by HPLC and MALDI-TOF mass spectrometry analyses of a peptide sample cleaved from a tenth of the total resin (≈1 μmol). As a fluorescent peptide lipoconjugate, the MHC class II binding peptide LGQQEPFPPEQPYPQPEPF was synthesized on the Pam_3Cys-loaded resin with two 6-aminohexanoic acid spacers at the C terminus of the peptide and labeled with carboxyfluorescein at the N terminus.

Cell culture: MEL-JUSO cells stably transfected with a fusion protein of the human MHC class II molecule HLA-DRB*0101 with the green fluorescent protein (GFP)[27] were grown in a 5% CO_2 humidified atmosphere at 37 °C in Iscove's modified Dulbecco's medium (IMDM) with Glutamax-1, supplemented with 10% fetal calf serum, penicillin (100 U mL^{-1}), streptomycin (0.1 mg mL^{-1}; Boehringer, Mannheim, Germany), and geniticin (400 μg mL^{-1}; Gibco, BRL, Eggenstein, Germany). The cells were obtained from J. J. C. Neefjes, Netherlands Cancer Institute, Amsterdam, The Netherlands. The human T cell lymphoma cell line Jurkat ACC 282 was obtained from the DSMZ (German collection of microorganisms and cell cultures, Braunschweig, Germany). The cells were cultured in RPMI-1640 medium with 10% inactivated fetal calf serum (Pan, Aidenbach, Germany) in a 5% CO_2 humidified atmosphere at 37 °C.

Microstructured functionalization of surfaces: Stock solutions (1 mg mL^{-1}) of the lipoconjugates in tertiary butyl alcohol/water (4:1) were diluted into deionized water to the final concentrations indicated for each experiment. Drops of these solutions (0.5 μL) were manually pipetted onto silanized coverslips and dried at ambient conditions. For immunofluorescence and streptavidin binding, the coverslips were blocked with 1% BSA in 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffered saline (HBS; 10 mM Na-HEPES, 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) for 30 min at room temperature. Incubation of the coverslip with a mixture of monoclonal murine α-human-myc-IgG antibodies (0.1 mg mL^{-1}; Dianova, Hamburg, Germany) and streptavidin (0.1 mg mL^{-1}; $Alexa_{546}$-labeled streptavidin, Molecular Probes, Leiden, The Netherlands, diluted 1:4 with unlabeled streptavidin, Dianova) was carried out for 30 min at RT. An $Alexa_{488}$-labeled goat anti-mouse IgG antibody (Dianova, Hamburg, Germany) diluted to 2 μg mL^{-1} in HBS was employed as a secondary antibody. After each incubation, the coverslips were washed with HBS. For microscopy the coverslips were mounted in a custom-made coverslip holder. Functionalization of coverslips for cellular analyses proceeded in a similar way. For the determination of the resistance versus cell growth, the coverslips were incubated with a mixture of unlabeled/labeled streptavidin as described above, followed by incubation with MHC class II expressing cells seeded at a density of 15 000 cm^{-2}. The coverslips were used for microscopy the next day.

For microstructured immobilization of Jurkat cells a streptavidin-functionalized surface was incubated for 30 min at room temperature with a biotin anti-human CD3ε antibody (5 μg mL^{-1}; Pharmingen/BD Bioscience, Europe) then washed three times with HBS. A suspension of Jurkat cells in RPMI-1640 medium (Gibco BRL/Life Technologies, Europe) was incubated for 30 min at 37 °C in a 0.5% CO_2 humidified atmosphere on the microstructured surface.

Nanopipetting: For the generation of microarrays with subnanoliter volumes a Tecan Genesis NPS 100/8 nanopipetting device (Tecan, Männedorf, Switzerland) was used. Solutions of lipoconjugates and silanized surfaces were treated in the same way as those used for manual pipetting. The pipetted volumes increased from 0.5 – 5.0 nl in steps of 0.5 nl. The same volumes and concentrations of aqueous solutions of the biotin succimidyl ester (6-((6-((biotinoyl)amino) hexanoyl)amino)hexanoic acid, sulfosuccinimidyl ester, sodium salt (biotin-XX, SSE); Molecular Probes, Leiden, The Netherlands) were pipetted for the amino-functionalized surfaces.

Atomic force microscopy: The samples were imaged in air with a Nanoscope III atomic force microscope (Digital Instruments, Santa Barbara, CA). Silicon tips (Nanosensors, Wetzlar, Germany) were used in tapping mode at a resonance frequency of 320 kHz. The line scan frequency was 2 Hz. The images were left unfiltered except for slope removal along each scan line to level the image.

Fluorescence microscopy and fluorescence correlation spectroscopy: Fluorescence images were recorded on an inverted LSM510 laser scanning microscope (Carl Zeiss, Göttingen, Germany). Fluorescein and $Alexa_{488}$ were excited at 488 nm and fluorescence detected through a BP505 – 550 band-pass filter; $Alexa_{546}$ was excited at 543 nm and fluorescence detected with an LP560 long-pass filter. For simultaneous dual channel recordings of fluorescein and the indocyanine dye Cy5, a BP505 – 550 band-pass filter and a LP650 long-pass filter were used for fluorescein and Cy5 detection, respectively.

Fluorescence correlation measurements were performed on a fluorescence correlation microscope based on a Zeiss Axiovert 35 inverted microscope with a C-Apochromat 40×1.2 W objective.[37] Fluorescence was excited at 488 nm and detected with a 500DRLP dichroic mirror (Omega Optical, Brattleboro, VT) and a 515 – 545 detection filter (Delta Light & Optics, Lyngby, Denmark). Power densities in the detection focus were $\approx 8\ kW\,cm^{-2}$. Autocorrelation functions were generated on-line with an ALV-5000/E autocorrelator board (ALV-Laservertriebsgesellschaft, Langen, Germany) and fitted off-line with Igor Pro (WaveMetrics, Lake Oswego, OR) to an autocorrelation function including one diffusional and one reaction term for fluorescein and the EGFP variant of the green fluorescent protein.[38] A stock solution ($2\ mg\,mL^{-1}$) of the fluorescein-labeled Pam_3Cys – peptide lipoconjugate was diluted 1:50 in water and treated in an ultrasound bath at room temperature for 10 min. A series of autocorrelation measurements was acquired over 60 s each. Reference measurements were carried out for fluorescein in water and EGFP in HBS.

Ca^{2+} imaging: For Ca^{2+} imaging Jurkat cells were taken up in phosphate-buffered saline (PBS) containing 0.1 % BSA/5 mM glucose in a density of $1 \times 10^6\ mL^{-1}$. The calcium-dye Fluo-3 (TEF LABS, Austin Texas, USA) was added in dimethylsulfoxide (DMSO) at a final concentration of 5 μM (stock solution 2 mM). Incubation took place for 40 minutes at room temperature in the dark. The cells were washed three times with PBS, taken up in HBS, and transferred onto coverslips functionalized with anti-CD3ε antibody as described above. The calcium signal was measured by confocal laser scanning microscopy by using an LSM510 apparatus with a C-Apochromat 63×1.2 W objective at nonconfocal settings. First the focus was adjusted to the focal plane of the spots and then the cells were added to a final density of $5 \times 10^5\ mL^{-1}$ in 0.5 mL total volume. A time series was recorded for 10 minutes with image acquisition every 10 seconds.

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