In Situ Peptide-Modified Supported Lipid Bilayers for Controlled Cell Attachment

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In Situ Peptide-Modified Supported Lipid Bilayers for Controlled Cell Attachment

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The control of cellular interactions with engineered materials is critical for the development of cell-integrated biochips used in cell-based sensors, "lab-on-a-chip" bioanalytical systems, and artificial neuronal networks, as well as medical implants and functional biomaterial scaffolds for tissue engineering. Supported lipid bilayers offer efficient reduction of nonspecific cell and protein binding and, if selectively functionalized, constitute one attractive approach to surface modification strategies of materials used in such devices. The present work describes the in situ modification of supported lipid bilayers through the coupling of a cysteine-terminated peptide to thiol-reactive maleimido lipids incorporated in the bilayer. The accumulation of peptide at the lipid bilayer interface was monitored by the quartz crystal microbalance technique with dissipation monitoring (QCM-D). Coupling of the peptide could be detected by QCM-D with a high signal-to-noise ratio despite its low molecular weight (2 kDa), primarily because the mass uptake included both peptide and the water associated to it. Lipid bilayers that were modified with the cysteine-terminated IKVAV-containing peptide promoted the binding of anti-IKVAV antibodies, as well as the attachment of PC12 cells, which express a membrane receptor for the IKVAV sequence. Very low nonspecific binding of peptides, proteins, and the cells was observed on nonfunctionalized lipid bilayers. Similarly, IKVAV-functionalized lipid bilayers were resistant to serum protein adsorption as well as the binding of non-IKVAV-specific antibodies. QCM-D and fluorescence recovery after photobleaching revealed that the lipid bilayers persisted under all the experimental conditions used for cell attachment, including staining and fixation. Thus, the described lipid-based surface modification is highly relevant for the development of controlled cell-attachment substrates and can even be applicable for patterning cell attachment because lipid-bilayer formation by vesicle fusion is material-specific.

Introduction

The rapidly increasing knowledge of the mechanisms of cell−cell interactions and cell−substrate interactions provides a strong driving force for advances in biotechnology and biomaterials research and development. For example, the emergence of cell sensors for the detection of toxic substances or for high throughput screening of new pharmaceutical drugs relies on the localization of cells in integrated networks on silicon- or polymer-based microfabricated circuits or "lab-on-a-chip" analytical devices. The same is true for artificial neuronal networks that are used to study cell−cell communication and signal transduction pathways through patterned neurons coupled to a network of electrodes. New chemical and structural surface modifications on medical implants, which are intended to influence cell behavior at the interface after implantation, are published or sold commercially for clinical use at an accelerating pace. The present development of tissue-engineered implants, which aim to help the body repair itself, will have great impact on difficulties related to the failure of alloplastic implants within the lifetime of the patient.[1,2] The control of cell interactions at artificial material surfaces is critical for the success of advanced tissue engineering, where bioresorbable material scaffolds support cell attachment and growth, leading to extracellular matrix production and the generation of new tissue either in vitro or in situ.

The present study focuses on the interplay between cells and engineered cell-culture substrates, in particular the modification of the substrate surface by immobilization of extracellular matrix ligands as a means to promote cell attachment. Several strategies for immobilizing cell attachment ligands (commonly proteins, peptides, or carbohydrates) to a wide variety of substrate materials have been published previously. For a brief review, the reader is referred to Tirell et al.[3] The immobilization procedure typically involves adsorption[4] or covalent coupling (e.g., thiol-based couplings[5] or photoinduced reactions[6]) to a solid support. Alternatively, the ligand can be bound via strong biospecific interactions (e.g., biotin−streptavidin[7]). In contrast, the use of weaker interactions, such as that between metal ion chelating compounds (e.g., histidine-tagged proteins), is less attractive for cell applications.

As an attractive modification of a material by cell-signaling molecules, the present study proposes a lipid-based strategy for surface modification, where the bioactive molecules are immobilized to a bioinert, fluidic lipid bilayer covering the material surface using a covalent coupling reaction. The use of supported lipid bilayers exposing biospecific ligands in cell-attachment studies represents an increase in complexity compared to previous

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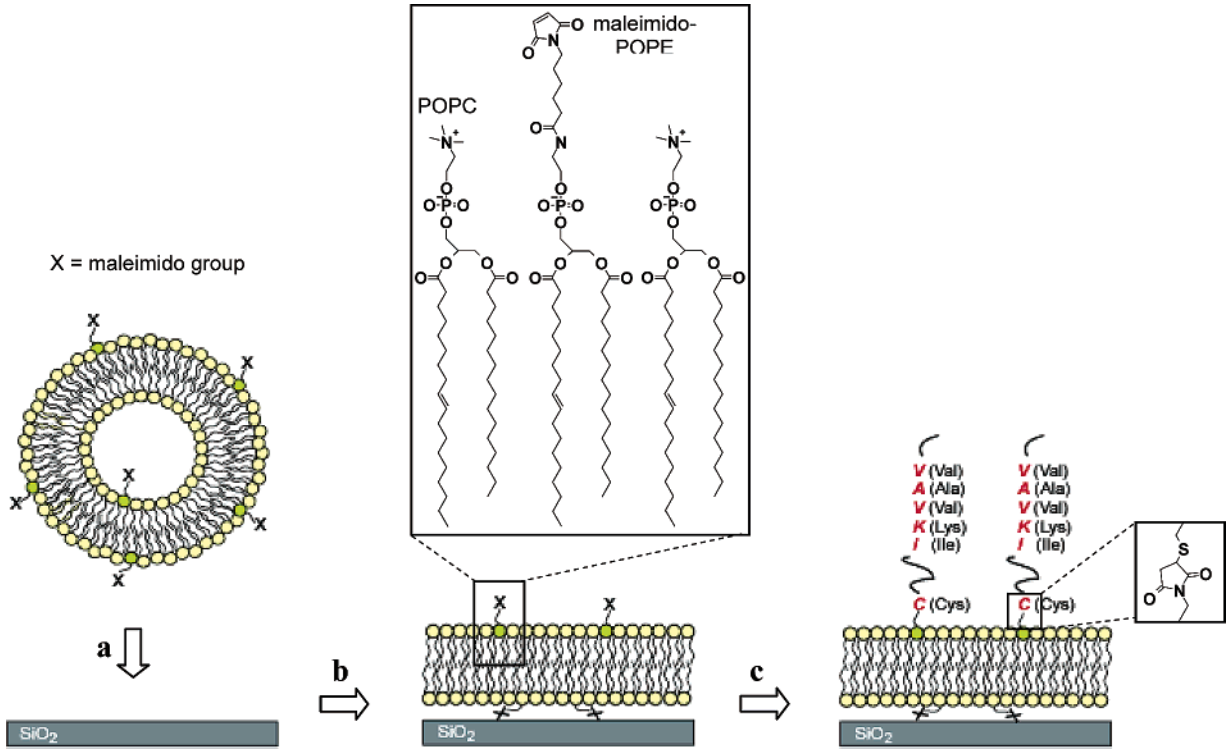

Figure 1. Schematic illustration of the surface modification strategy. (a) Lipid vesicles consisting of a mixture of maleimido-POPE and POPC are adsorbed from a solution onto a SiO_2 substrate. (b) Vesicles rupture and fuse at the surface, forming supported lipid bilayers on the solid support. (c) The maleimido lipid subsequently reacts with the cysteine-terminated peptide, which results in the covalent binding of IKVAV-containing peptide sequences to the bilayer.

studies demonstrating the cell repellant properties of unmodified supported lipid bilayers.[8−10] The resistance to nonspecific cell adhesion parallels nondenaturing conditions for immobilized molecules, and as a rule of thumb, surfaces that resist protein adsorption will also be resistant to cell adhesion.[11] Therefore, it is not unexpected that the fluidic, liquid crystalline supported phosphatidyl choline lipid membranes have also shown very promising results with respect to protein adsorption,[12] even from complex biological mixtures.[13] The efficient reduction of nonspecific binding, in combination with the possibility to include a small fraction of chemically modified lipids designed for specific interactions or coupling reactions, makes supported phophatidyl choline lipid bilayers suitable to achieve selective bioreactivity at the cell−material interface. In addition, supported lipid bilayers will be useful for model experiments where cell interactions with immobilized ligands are studied at the molecular level.

As a model system for the present study, a well-known interaction between a peptide signal recognition motif and nerve cells was sought for. In earlier work using synthetic peptides, a large number of peptide sequences from the extracellular matrix glycoprotein laminin-1 has been attributed to cell adhesion or neurite outgrowth activity.[14] Among them, IKVAV-containing sequences have been found to promote the cell adhesion and neurite outgrowth of PC12 cells.[4,15,16] This interaction was chosen as the model interaction in this study because of its relevance for neural cell tissue engineering, as well as the availability of both peptide and cells from commercial sources. The synthetic 19-mer IKVAV-containing peptide used (CS-RARKQAAS**IKVAV**SADR) had a single cysteine at the C-terminal end, providing a unique reactive functional group for covalent coupling to thiol-reactive lipid headgroups (here maleimido groups) (Figure 1). The specific in situ coupling of antibody fragments via free cysteines (exposing −SH groups) to supported lipid bilayers containing thiol-reactive maleimido and dithiopyridyl phospholipids has been demonstrated.[17,18] However, the objectives of the present study were to use this approach to immobilize the relatively smaller IKVAV-containing peptide to supported lipid bilayers prepared on SiO_2 substrates by the lipid vesicle fusion technique and to evaluate the performance of such a lipid-based surface modification in PC12-cell-attachment experiments. The lipid bilayer surface modification protocol was established using the quartz crystal microbalance with dissipation monitoring[19] (QCM-D) technique, in combination with fluorescence microscopy. The merit of the QCM-D technique lies primarily in the combined information

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contained in the simultaneously measured changes in the resonance frequency shift, f, and energy dissipation shift, D. For a given added mass, Δm, there is a proportional change in f, which increases linearly with the overtone number, n (=1, 3, ...):[20]

$$\Delta m_{\text{Sauerbrey}} = \rho_{\text{film}}\delta_{\text{film}} = \frac{C_{\text{QCM}}}{n}\Delta f \qquad (1)$$

where ρ_{film} and δ_{film} are the effective density and the film thickness, respectively, and C_{QCM} is the mass-sensitivity constant [17.7 ng/(cm^2 Hz) for the 5 MHz crystals used in the present study]. However, for the QCM measurements conducted in aqueous solution, water is generally coupled as an additional mass via direct hydration, viscous drag, or entrapment in cavities in the adsorbed film.[21] This means, in turn, that the layer is essentially sensed as composed of both adsorbed macromolecules and coupled water.[22] Fluorescence microscopy is a valuable complementary technique to QCM-D for the type of system investigated in this study, because lateral mobility of the lipid molecules in a supported bilayer can be established in fluorescence recovery after photobleaching (FRAP) experiments.

Results and Discussion

The lipid-based surface modification developed in this work consists of supported lipid bilayers formed by the fusion of vesicles onto SiO_2 and subsequent modification by immobilization of a peptide. In the first part of the study, the lipid-based surface modification was established and characterized using the QCM-D technique. The results from these experiments formed the basis for the second part of the study, namely, cell-attachment experiments using SiO_2 substrates modified by supported lipid bilayers that either presented or did not present the cell-attachment peptide.

Establishment of the Lipid-Based Surface Modification. Maleimidopalmitoyloleylglycerophosphoethanolamine (maleimido-POPE) was synthesized as is described in the experimental section. Mixed maleimido-POPE and palmitoyloleylglycerophosphocholine (POPC) vesicles were prepared by extrusion. The interaction between the maleimido vesicles and SiO_2-coated substrates was studied in real time by QCM-D. The QCM-D technique is particularly useful to distinguish between the adsorption of intact lipid vesicles and the formation of supported lipid bilayers on the surface of the quartz crystal because of the simultaneous measurement of changes in the resonance frequency shift, f (related to coupled mass; $\Delta f < 0$ corresponds to adsorption of mass), and energy dissipation shift, D (related to viscous losses in adsorbed films; $\Delta D > 0$ corresponds to a more viscous film). Nonruptured, adsorbed vesicles at the surface induce changes both in f and in D, whereas the planar supported bilayer contributes only to changes in f and does not induce significant viscous losses (i.e., changes in D). Previously, sonicated egg phosphatidyl choline vesicles have been shown to form bilayers on SiO_2-coated quartz crystals ($\Delta f_{n=3} \approx -78$ Hz, $\Delta D_{n=3} \approx 0$, corresponding to an effective thickness of ~4 nm using eq 1 and a density of 1.1 g/cm^3).[25−27] In this study, as is shown in Figure 2 and Table 1, plain POPC vesicles gave similar results [$\Delta f_{n=3} = -83 \pm 2$ Hz, $\Delta D_{n=3} = (0.43 \pm 0.2) \times 10^{-6}$].[28] Likewise, vesicles with a small fraction of maleimido-POPE (5%) fused readily [Figure 2, Table 1; $\Delta f_{n=3} = -82 \pm 3$ Hz, $\Delta D_{n=3} = (0.42 \pm 0.1) \times 10^{-6}$]. It is clear that such low concentrations of maleimido lipids do not significantly alter the vesicle rupture and fusion process compared to plain POPC vesicles. In contrast, maleimido vesicles with a large fraction (50%) of the maleimido lipid did not fuse onto the SiO_2 but adsorbed as intact vesicles (not shown).

The maleimido-supported bilayer was subjected to the cysteine-terminated, IKVAV-containing peptide. In favor of the expected formation of covalent bonds between the peptide and the maleimido lipids incorporated in the bilayer, QCM-D data showed the accumulation of mass at the sensor surface (Figure 2, Table 1), and no material dissociated from the surface upon rinsing. Note that despite the low molecular weight of the peptide (2 kDa), pronounced effects on both Δf and ΔD were observed upon coupling. When simple assumptions were used,[30] the maximum decrease in Δf upon coupling of the peptide estimated from the Sauerbray equation[20] was $\Delta f_{n=3} = -8$ Hz. The large discrepancy between this value and the observed value ($\Delta f_{n=3} \sim -20$ Hz) is primarily due to water associated to the peptide,[21] rendering an effective thickness of ~1 nm using eq 1 and a density close to that of water.[31] This value is in good agreement with the expected coiled conformation of the peptide. Thus, similarly to the response from DNA strands attached to a lipid bilayer via streptavidin,[32] the detection of peptide was greatly enhanced by the surrounding water. In control experi-

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(22) Because the amount of coupled water generally varies depending on the nature of the adsorbed layers, this complicates the use of Δf for quantitative estimations of the amount of adsorbed biomolecules. However, at a high surface coverage, it has been shown that essentially all water between adsorbed molecules is coupled as a mass,[23] which motivates the use of Δf for estimations of the hydrodynamic thickness, δ_{film}, of the formed layers, rather than the adsorbed amount, given that the density of the adsorbed layer is known. This generally requires an independent measure of the "dry mass" of the adsorbed layer, but even without such data, the error induced by choosing a density somewhere between that of water (~1 g/cm^3) and that of the analyzed biomolecules (1.1 g/cm^3 < ρ_{film} < 1.4 g/cm^3) will not be very large. One must be aware, however, that if the adsorption-induced ΔD is large, eq 1 might no longer be valid.[23] In such situations, a Voight−Kelvin-based model,[24] in which the adsorbed film is represented by not only an effective thickness and density but also a complex shear modulus, should be applied. In the present work, the induced ΔD per coupled mass ($\Delta D \sim 0$) is in the regime where the Sauerbrey relation holds for the complete supported lipid bilayer, while the mass estimated using eq 1 is underestimated by not more than 10% for any system. Because the conclusions drawn from the present work are not dependent on very precise quantification of the amount of adsorbed nonruptured vesicles, this type of modeling is, however, not explicitly included.

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(28) There will always be a small, remaining fraction of unruptured vesicles present together with the bilayer. Therefore, the asymptotic values of Δf and ΔD depend on the size of the vesicles. The extruded vesicles used here were somewhat larger (diameter ~80 nm) than the sonicated vesicles used in the previous studies (diameter ~30 nm).[29]

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(30) The cross-sectional area of a lipid molecule in the bilayer is 70 Å^2, fraction of maleimido lipids is 2 × 5% (including both leaflets of the lipid bilayer), and molecular weight of the peptide is 2017 g/mol.

(31) A density close to that of water was chosen because the induced energy dissipation was high, which generally indicates a high degree of coupled water.

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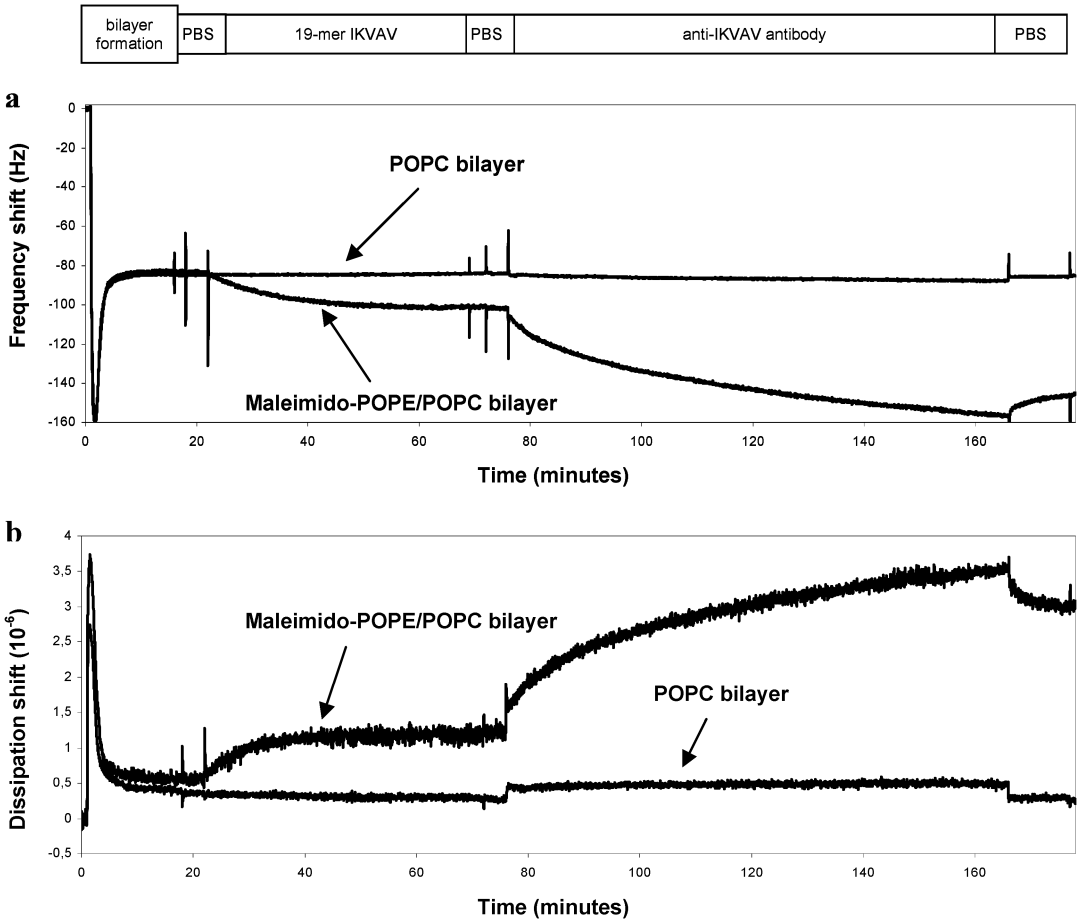

Figure 2. QCM-D data showing shifts in (a) frequency and (b) dissipation during the formation of a supported lipid bilayer on SiO_2 by vesicle fusion using either plain POPC vesicles or maleimido vesicles (5% maleimido-POPE), followed by the injection of the cysteine-terminated IKVAV-containing peptide, and subsequent interaction with 1% anti-IKVAV antibody serum. The boxes at the top of the figure indicate each step in the procedure and span over the duration of each step.

Table 1. Representative QCM-D Frequency and Dissipation Shifts Obtained after Bilayer Formation and after Exposure of Bilayers to Various Peptides and Proteins[a]

maleimido vesicles	+ 19-mer IKVAV	+ anti-IKVAV
$\Delta f_{n=3} = -83$ Hz $\Delta D_{n=3} = 0.55 \times 10^{-6}$	$\Delta f_{n=3} = -18$ Hz $\Delta D_{n=3} = 0.65 \times 10^{-6}$	$\Delta f_{n=3} = -56$ Hz $\Delta D_{n=3}$[b] $= 2.35 \times 10^{-6}$
maleimido vesicles	+ 19-mer IKVAV	+ anti-laminin
$\Delta f_{n=3} = -84$ Hz $\Delta D_{n=3} = 0.50 \times 10^{-6}$	$\Delta f_{n=3} = -16$ Hz $\Delta D_{n=3} = 0.75 \times 10^{-6}$	$\Delta f_{n=3} = 0$ Hz $\Delta D_{n=3} = 0$
maleimido vesicles	+ 19-mer IKVAV	+ serum
$\Delta f_{n=3} = -81$ Hz $\Delta D_{n=3} = 0.55 \times 10^{-6}$	$\Delta f_{n=3} = -25$ Hz $\Delta D_{n=3} = 1.10 \times 10^{-6}$	$\Delta f_{n=3} = -2$ Hz $\Delta D_{n=3} = 0.15 \times 10^{-6}$
maleimido vesicles	+ SIKVAV	+ anti-IKVAV
$\Delta f_{n=3} = -82$ Hz $\Delta D_{n=3} = 0.20 \times 10^{-6}$	$\Delta f_{n=3} = -0.5$ Hz $\Delta D_{n=3} = 0$	$\Delta f_{n=3} = 0$ Hz $\Delta D_{n=3} = 0$
POPC vesicles	+ 19-mer IKVAV	+ anti-IKVAV
$\Delta f_{n=3} = -85$ Hz $\Delta D_{n=3} = 0.40 \times 10^{-6}$	$\Delta f_{n=3} = 0.5$ Hz $\Delta D_{n=3} = -0.05 \times 10^{-6}$	$\Delta f_{n=3} = -1.5$ Hz $\Delta D_{n=3} = 0$

[a] In general, the QCM-D measurements were repeated under the same or similar conditions. The experiments were highly reproducible, with the exception of the oxidation-sensitive coupling of the cysteine-terminated peptide to the maleimido bilayers (see text). [b] Values obtained 90 min after the addition of the antibody.

ments, no binding of peptide was observed to plain POPC bilayers (Figure 2, Table 1). The presence and the appropriate exposure of the peptide were further confirmed in experiments with anti-IKVAV antibody serum[33] (Figure 2, Table 1). The relatively low decrease in f for the antibody with a molecular weight being more than a factor of 20 heavier than that of the peptide is attributed to a significantly lower amount of coupled water in this case. Again, control experiments with plain POPC and underivatized maleimido-supported bilayers showed no nonspecific binding of the anti-IKVAV antibody serum (Figure 2, Table 1). The high resistance of the peptide-functionalized bilayer to nonspecific protein adsorption was shown in experiments with serum which did not contain the anti- IKVAV antibodies (Table 1). Similarly, purified polyclonal antilaminin antibodies did not bind to the IKVAV-exposing bilayers (Table 1). In addition, a non-cysteine-containing peptide (SIKVAV) did not accumulate at the maleimido supported bilayer (Table 1). The results with purified polyclonal anti-laminin antibodies were also tested but did not bind to the IKVAV-exposing bilayers (Table 1). The results with respect to nonspecific interactions were very encouraging and similar to, or better than, the in situ QCM data on the maleimidodipalmitoylglycerophosphoethanolamine/dipalmitoylglycerophosphocholine floating monolayer films (as opposed to transferred films) reported earlier.[18,34,35] The nonspecific binding is likely to reflect the quality of the lipid film. The vesicle fusion technique is an attractive method for achieving

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high-quality membranes,[12] on the condition that vesicles of the desired composition fuse.

A difficulty when working with thiols is the spontaneous formation of disulfides by oxidation in air. Indeed, if the cysteine-terminated peptide was not handled under an inert atmosphere, the yield of the reaction with the maleimido-supported lipid bilayer was reduced. The accumulation of peptide at the bilayer completely ceased in some experiments, strongly indicating that the peptide could only bind to the bilayer in its reduced form, hence, via covalent bonds, and that it did not associate to the bilayer via noncovalent interactions.

PC12 Cell Attachment to 19-mer-IKVAV-Modified Supported Lipid Bilayers. Cell-attachment experiments were motivated by the specificity in the coupling of the IKVAV-containing peptide to the maleimido bilayer and the subsequent binding of the anti-IKVAV antibody, as well as the nonspecific interactions below the detection limit. In accordance with the established interaction between PC12 cells and IKVAV-containing peptide fragments, the attachment of PC12 cells to the IKVAV-modified supported lipid bilayer was greatly enhanced in comparison with that of plain POPC bilayers, as well as with that of maleimido bilayers that had not been subjected to the IKVAV peptide (Figure 3, Table 2). The results with respect to nonspecific cell attachment were very promising: very few cells attached to the nonfunctionalized supported bilayers (Figure 3, Table 2). A clean SiO_2 substrate was included in the series of experiments as a control cell-culture substrate and induced, via nonspecific interactions, the attachment of more cells than the IKVAV-modified bilayer. The attachment of cells to SiO_2 is to be expected because silicon oxide (e.g., glass) is a common cell-culture substrate that generally promotes cell attachment. Silicon oxide typically coats silicon wafers used for microfabricated circuits. However, in many biochip applications it is important to have an inert background to achieve controlled cell attachment.

The great advantage of the IKVAV-modified lipid bilayers is the high degree of control of the exposure of the cell recognition ligand. However, a drawback of supported lipid membranes is their low stability under certain conditions, such as the presence of detergents or lipases, or upon drying. Fluid lipid membranes, therefore, may not be suitable for cell culture for longer periods of time, at least not without stabilization. Approaches to more stable lipid membranes are being developed, using, for example, lateral cross-linking photopolymerization reactions[35,36] or stronger interactions between the support and the supported lipid bilayer.[37,38] In the present work, additional control experiments were performed to ensure that the supported lipid bilayers withstood the cell-culture medium and fixation procedures used. QCM-D data showed that the characteristic Δf and ΔD values for the bilayer were not altered by exposure to any of the solutions (Figure 4a). After rinsing with phosphate-buffered saline (PBS), the Δf and ΔD curves return to the values for the bilayer. The experiment was carried out using vesicles doped with the maleimido lipid, and it was repeated in a separate liquid cell that could be imaged under the microscope with POPC vesicles doped with a fluorescent dye. As was expected from the QCM-D data, the lipid bilayer persisted and maintained its lateral fluidity, as was proven by FRAP experiments (Figure 4b).

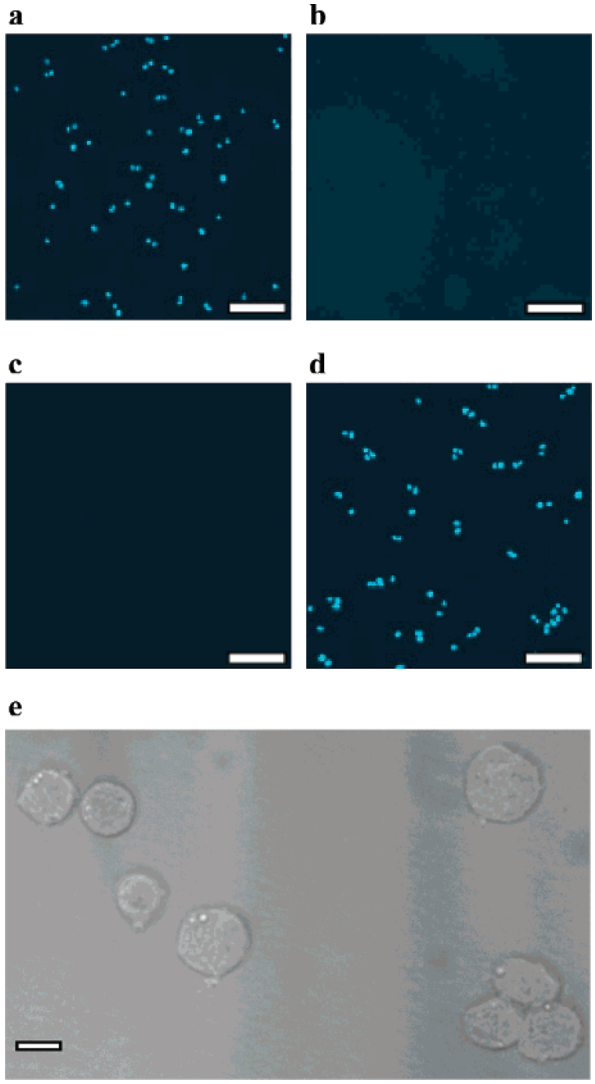

Figure 3. PC12 cell attachment to various substrates after 1 h of incubation. Fluorescence microscopy images of cells stained for the nuclei (DAPI) on (a) unmodified SiO_2, (b) SiO_2 modified by the fusion of plain POPC vesicles, (c) SiO_2 modified by the fusion of maleimido vesicles, (d) same as that in part c but further modified with the 19-mer-IKVAV peptide. (e) Bright field microscopy image of cells attached to a 19-mer-IKVAV-peptide modified bilayer after 1 h. The scale bars in parts a−d are 100 μm. The scale bar in part e is 10 μm.

Table 2. PC12 Cell-Attachment to 19-mer-IKVAV-Modified Supported Lipid Bilayers and Control Surfaces

surface modification	experiment 1 cells/mm^2	experiment 2 cells/mm^2
SiO_2	249 ± 16.7	175 ± 10.2
SiO_2, POPC	1 ± 0.7	2 ± 2.4
SiO_2, POPC/maleimido-POPE	1 ± 1.0[a]	0 ± 0.1
SiO_2, POPC/maleimido-POPE, 19-mer IKVAV	157 ± 22.8	136 ± 25.9

[a] Performed on another occasion.

A question can be raised about the specificity of the interaction between the PC12 cells and the IKVAV-modified supported lipid bilayer. However, in this study we are satisfied by the fact that we can alter the interactions of cells with the lipid bilayer by modification with peptides. On the basis of previous studies with IKVAV-modified surfaces and PC12 cells, the interaction is likely to be specific.[4] One can argue that the functional

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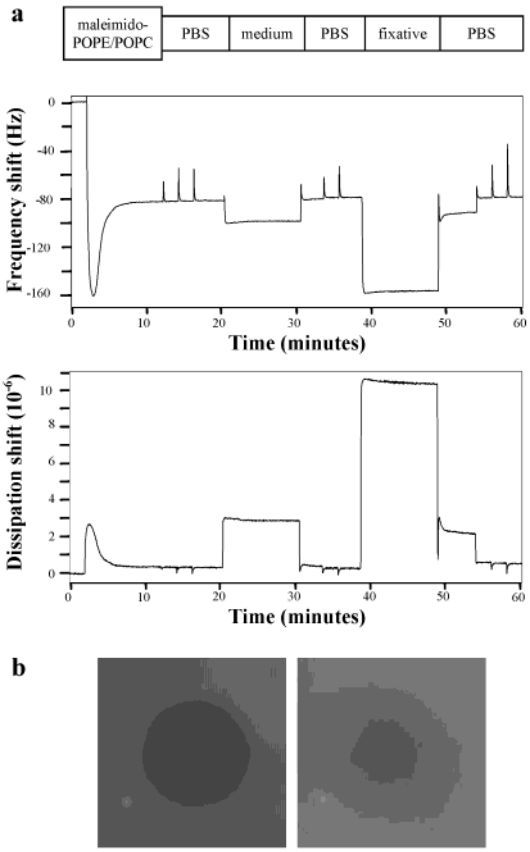

Figure 4. (a) QCM-D data showing the effect of subjecting the supported maleimido-lipid bilayer to the cell-culture medium and the cell-fixation treatment. (b) FRAP of a similarly treated fluorescent lipid bilayer. The left image was taken immediately after bleaching; the right image was taken after 15 min.

proof of neurite outgrowth would show specificity in the interaction of the cells with the modified bilayer. However, in this work we have chosen to focus on the initial cell attachment, and, therefore, the cells were not treated with nerve growth factors (required for neurite outgrowth)[4] prior to the seeding.

Future Perspectives. The strategy for the lipid-based surface modification described in this work is well-suited for the further development of more elaborate cell-attachment substrates, primarily with respect to different ligands, with the only requirement being the presence of a free −SH group. In addition, the role of steric effects, such as the potential need of a spacer between the surface and the immobilized biomolecules,[39] as well as the density of immobilized molecules,[40,41] can be studied in greater detail. Furthermore, the microenvironment of the immobilized ligands can be varied by changing the composition of the lipid bilayer, thus affecting, for example, charge and fluidity effects. The supported lipid bilayer is a simple mimic of cell membranes. We anticipate that, gradually, supported lipid bilayers will make up more and more valuable model systems for elucidating the details of cell signaling interactions.

Furthermore, significant progress has recently been made toward patterning lipid membranes at surfaces.[42] Similarly, patterns of surface-immobilized peptides have been prepared,[11] and recently, a printed peptide chip has been described, suitable for high throughput assays of cell adhesion and function.[43] Thus, the type of lipid bilayer surface modification presented above will be valuable in the design of various microfabricated substrates for patterned cell attachment. In addition, we propose variants of the presented lipid-based surface modification to be used for the modification of biodegradable polymeric scaffolds for the guidance of tissue regeneration. The combination of phospholipids and polymers exposing phosphorylcholine groups (acting as tethers for the lipid membranes) has been investigated with respect to thromogenicity.[44] The field of applications for similarly prepared biodegradable materials is currently wide open.

Conclusions

The present work describes the in situ modification of supported lipid bilayers through the coupling of a cysteine-terminated peptide to thiol-reactive maleimido lipids incorporated in the bilayer. The accumulation of peptide at the lipid bilayer interface was monitored by QCM-D, and the signal was greatly enhanced by the simultaneous detection of the peptide and water associated with it. The lipid bilayers that were modified with the IKVAV-containing peptide promoted the binding of anti-IKVAV antibodies, as well as the attachment of PC12 cells. QCM-D as well as FRAP results showed that the lipid bilayers persisted under cell-attachment and fixation conditions. Very satisfactory results were achieved, both with proteins and with cells, with respect to nonspecific interaction with lipid bilayers that did not expose the IKVAV-containing peptide, as well as nonspecific binding of proteins and antibodies to the IKVAV-modified bilayer. The surface modification method is generic in the sense that it should be easily extended to a broad range of biomolecules exposing −SH groups. Thus, the presented lipid surface modification will be useful in the patterning of substrates for controlled cell attachment.

Experimental Section

Unless otherwise stated, chemicals were obtained from commercial sources and used without further purification. Water was deionized (resistivity > 18 MΩ cm) and purified (Milli-Q unit, Millipore, France). PBS was prepared from tablets (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, and pH = 7.4). All buffers were filtered and degassed. The peptides CSRARKQAAS**IKVAV**SADR (Sigma; C6171) and SIKVAV (Bachem, Germany, Bubendorf; H2684) were dissolved in a phosphate buffer (10 mM, pH = 7.0) to 1 mg/mL and frozen in aliquots. Care was taken to keep the IKVAV-containing peptide under N_2 to avoid oxidation. The rabbit anti-IKVAV antibody serum (no. 175, raised against CSRARKQAASIKVAVSADR)[33] was a gift (Hynda K. Kleinman, NIDCR, NIH, Bethesda, MD). The anti-IKVAV serum was frozen in aliquots. Purified anti-laminin antibodies (Sigma; L9393) were refrigerated. 1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) was from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Lipids were stored at −20 °C as powders or dissolved in chloroform. 3-Octadecyl-2-[3-(3-octadecyl-2(3*H*)-benzoxazolylidene)-1-propenyl] (DiO; Molecular Probes, Eugene, OR, U.S.A.; D-275) was dissolved and diluted to 1 mg/mL in dimethyl sulfoxide (DMSO) and stored frozen.

Chemical Synthesis. *N*-(6-Maleimidohexanoyl)-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (maleimido-POPE) was synthesized: 6-maleimidocaproic acid (16 mg, 0.08 mmol) was dissolved in $CHCl_3$ (5 mL) and cooled to 0 °C. *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (15 mg, 0.08 mmol) and *N*-hydroxysuccinimide (9 mg, 0.08 mmol) were added, and the mixture was stirred for 10 min. 1-Palmitoyl-2-oleoyl-

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sn-glycero-3-phosphoethanolamine (50 mg, 0.07 mmol) was added, and the mixture was stirred overnight. Concentration of the mixture, purification by flash column chromatography (5:1 $CHCl_3$/MeOH), and subsequent freeze-drying (DMSO) gave maleimido-POPE (33 mg, 52%) as a white powder. $R_f = 0.36$ (5:1 $CHCl_3$/MeOH); ^{1}H NMR ($CDCl_3$) δ 7.17 (s, 1H), 6.71 (s, 2H), 5.38 (m, 2H), 5.09 (q, $J = 4.4$ Hz, 1H), 4.37 (d, $J = 9.9$ Hz, 1H), 4.06 (dd, $J_1 = 12.1$ Hz, $J_2 = 7.1$ Hz, 1H), 3.95 (m, 5H), 3.42 (m, 4H), 2.25 (dd, $J_1 = 14.8$ Hz, $J_2 = 7.1$ Hz, 4H), 2.22 (t, $J = 7.7$ Hz, 2H), 2.00 (m, 4H), 1.60 (m, 10H), 1.28 (m, 44H), 0.85 (t, $J = 6.6$ Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 174.4, 173.7, 173.4, 171.0, 134.1, 130.0, 129.7, 70.4, 64.8, 63.9, 62.8, 40.0 (d), 37.6, 36.0, 34.3, 34.1, 32.0 (d), 29.8−29.1 (several peaks), 28.2, 27.3, 26.3, 25.4, 25.0, 24.9, 22.7, 14.1. Maldi-TOF-MS: calcd for MH^+, 911.6; found, 933.4 (MNa^+).

Preparation of Vesicles. POPC (5 mg) was dissolved in chloroform. When appropriate, 5% (w/w) maleimido-POPE (from 5 mg/mL stock solution in chloroform) or 0.5% (w/w) DiO (from 1 mg/mL stock solution in DMSO) was added. A lipid film was formed on the wall of a flask by evaporation of the solvent under a flow of N_2. The lipids were hydrated in 1 mL of PBS. The solution was extruded 11 times through a 100-nm filter followed by 11 times through a 30-nm filter (polycarbonate filter membranes were from Avanti Polar Lipids, Alabaster, AL, U.S.A.). The vesicle solutions were refrigerated under N_2 and used within 10 days. The vesicles (diluted to 0.5 mg/mL in PBS) were characterized by dynamic light scattering using a Brookhaven BI-90 particle sizer (Brookhaven Instrument Corp., NY). The average vesicle diameter calculated by the instrument was about 80 nm.

QCM-D Measurements. AT-cut quartz crystals (Q-sense AB, Göteborg, Sweden) with a fundamental frequency of 5 MHz, coated with SiO_2, were stored in a 10 mM sodium dodecyl sulfate solution between measurements. Prior to use, they were rinsed with water, dried with N_2, and treated with UV/ozone for 10 min. The cleaning procedure was performed twice. The QCM-D experiments were performed using a Q-sense D 300 system (Q-sense AB, Göteborg, Sweden). The sensed crystal area was approximately 20 mm^2, and the liquid volume interacting with the surface was about 80 μL. The samples were diluted to 1.5 mL (PBS), and the solution was temperature-stabilized for 2 min prior to the injection of 0.5 mL into the measurement chamber. The crystal was rinsed with buffer between each step. Injections can be seen in the QCM data plots as "spikes". The final concentration of the 19-mer-IKVAV-containing peptide and SIKVAV was 10 μg/mL and that of the vesicles was 200 μg/mL, and anti-laminin, anti-IKVAV-containing serum, and foetal calf serum were diluted 100 times.

Fluorescence Microscopy. Fluorescence microscopy was performed using a Zeiss Axioplan 2 E MOT microscope. FRAP experiments of DiO-doped bilayers were done on QCM crystals treated in a separate liquid cell, allowing imaging of the crystal. The liquid was exchanged from the top, and care was taken to keep the samples wet at all times. In a typical FRAP measurement, spots of approximately 180 μm in diameter were bleached for 15 s using a filter set appropriate to the DiO dye (excitation, 450−480 nm; emission, 510−560 nm). The first picture was taken immediately, and the lamp was switched off between pictures.

Cell Experiments. Silicon wafers covered with thermally grown SiO_2, cut in 6 × 6 mm^2 squares, were used as substrates. For cleaning, the samples were treated with UV/ozone (10 min), rinsed with water, dried (N_2), and treated a second time with UV/ozone (10 min). The samples were placed in the wells of a 48-well polystyrene microtiter plate (Greiner). At all times, there was at least 250 μL of liquid in each well. Surface modification was performed in a total volume of 500 μL using the same concentrations as those in the QCM-D measurements. The vesicles were allowed to interact with the samples for 40 min and the 19-mer-IKVAV-containing peptide for 1 h. The solution was exchanged to PBS between every step. The formation of bilayers on the substrates was verified by FRAP using DiO-doped vesicles (the sample was placed on an objective glass and covered with a cover slip, without dewetting).

PC12 cells (cell line obtained from L. A. Green, Department of Biochemistry, New York University School of Medicine, NY) were detached from the cell-culture flask and kept at 37 °C in a serum-free medium (Iscove's medium (Sigma) with 1% penicillin and streptomycin (PEST, Invitrogen), 1% L-glutamin (Invitrogen), 1% Na-pyruvat (Invitrogen)) until seeding (about 1 h). PBS in the wells was exchanged to the serum-free cell-culture medium. The area of the wells was 80 mm^2. Cells at a density of 300 cells/mm^2 (96 000 cells/mL) were seeded on four samples per surface modification. The samples were incubated for 1 h at 37 °C in a humidified atmosphere of 94% air and 6% CO_2. After incubation, the cell suspension was exchanged to PBS. The attached cells were fixed by formaldehyde (4%) in PBS (fixative solution), and the cell nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.1%) in PBS. The samples were then moved to objective glasses and covered by an antifading agent in glycerol (SlowFade Light Antifade Kit; Molecular Probes, Eugene, OR, U.S.A.; S-7461) and a cover slip. Five pictures (1300 × 1030 μm^2) were taken on each sample: one close to every corner and one in the center. Counting of the attached cells was done manually from the pictures, taking all nuclei into consideration. The average number of cells for each surface modification was calculated from the sample means.

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