Solid-Supported Biomimetic Membranes with Tailored Lipopolymer Tethers

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Summary: Stable lipid membranes with controlled substrate-membrane spacing can be prepared using well-defined lipopolymers as a tether. Based on the living cationic ring-opening polymerization of 2-methyl- or 2-ethyl-2-oxazoline, lipopolymers can be synthesized bearing a lipid head group as well as a silanol reactive coupling end group. Using a "grafting onto" procedure these polymers can form dense, brush like monolayers, whose layered structures can be obtained by x-ray reflectivity measurements. By transfer of a pre-organized monolayer that is followed by vesicle fusion, stable polymer supported lipid membranes can be prepared. The substrate-membrane spacing can be controlled via the degree of polymerization, while the lateral diffusion of lipids within the membrane depends on the density of polymer tethers. Preliminary experiments implied that the membrane with long (N = 40) polymer tethers could reside trans-membrane receptors homogeneously, suggesting a large potential of this strategy.

Keywords: lipopolymer; poly(2-alkyl-2-oxazoline); tethered lipid bilayer membrane

Introduction

The construction of supported lipid membranes on solid substrates has become a popular research topic within the last several years. Especially a planar configuration of the bilayer allows the application of numerous different surface and interface sensitive experiments to obtain a detailed insight of the plasma membrane structure and membrane associated transport processes. Such membrane models can not only be used to study the membrane behavior in detail but ultimatively to incorporate transmembrane proteins, attach lipid associated recognition and signaling sites and

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thus, investigate isolated functions and properties of biological cell membranes. Although several supported lipid membrane models were presented in the literature, a stable biomimetic membrane model, which enables meaningful investigation of various membrane associated diffusion and transport processes, is still at large. For membrane bilayers directly deposited onto a substrate the close proximity of a membrane and the solid support in the equilibrium state has a typical distance of 5 - 20 Å.[1] This thickness does not provide a sufficient internal water reservoir which results in nonspecific adsorption of incorporated membrane proteins to the substrate and their denaturing as well as a significant slower lateral diffusion in the lower lipid leaflet.[2] These limitations can be overcome by increasing the thickness of the lubricating water layer by introducing a hydrophilic polymer layer between the lipid membrane and the substrate as introduced by *Ringsdorf [3]* and *Sackmann*.[4] This polymer interlayer should also increase the mechanical stability of the supported membrane bilayer. E.g., black lipid membranes preserve their structure for only short time and are very sensitive to mechanical stress. Presumably, a good stabilization of the membrane can be obtained if the polymer interlayer is not only functioning as a cushion but also tethers the membrane to the supporting solid substrate. Such a polymer should feature a lipid head group for incorporation into the lipid layer by hydrophobic interactions and, on the other side, a chemical function for a permanent covalent attachment. This would form a defined brush-like lipopolymer monolayer, covalently bound to the surface and on the other side incorporated into the membrane (see Figure 1).

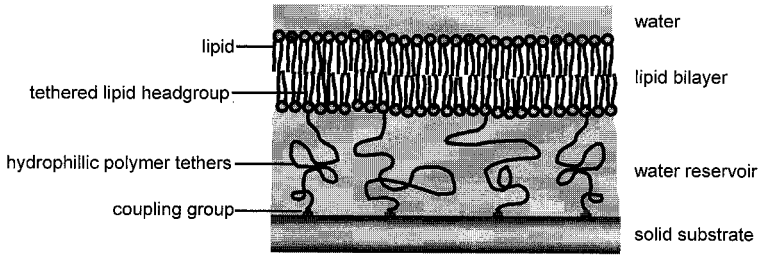


Figure 1. Solid-supported lipid bilayer tethered by lipopolymers.

However, if the grafting density of such polymer tethers is too high, the free mobility of the lipids in the under layer may be obstructed. So, for every application, a compromise between stability and fluidity needs to be found. Additionally, the type of polymer is crucial for the structure and

function of the supported membrane and incorporated proteins. The polymer must fulfill certain requirements: First, it should be a hydrophilic polymer which interacts neither with membrane proteins nor with the lipid bilayer. Regarding the stability aspects, the polymer should be hydrolytically stable. Since the introduction of the concept of polymer cushions/tethers between the lipid membrane and the solid support, different approaches for the construction of such membrane models were studied. *Tamm* et al.[5] used linear polyethylene glycol (PEG) with a lipid head group (lipopolymer) and a triethoxysilane end group. Although they varied the grafting density of the lipopolymer tethers, they limited their study to a single polymer length. *Naumann et al.* [6] reported on the use of poly(2-ethyl-2-oxazoline)s with a lipid head group. The lipopolymers were photochemically coupled to a self-assembled monolayer of benzophenone. Since the grafting reaction is unspecific, the morphology of the polymer cushion may vary strongly. *Schiller et al.*[7] used a tetrameric oligomer to avoid a helical lipopolymer backbone and a disulfide coupling group to ultra flat gold surfaces. Within this amphiphilic multilayer, the lipids displayed a good short-range mobility but a very low lipid diffusion coefficient over lager areas. The short oligomeric spacers were grafted at extremely high densities to maintain the layer morphology. Incorporation of larger proteins into such constructs might be difficult, if not impossible.

In order to obtain full control upon the grafting density of the polymer tether, the interaction between the lipopolymer and the membrane lipids as well as the fixation onto the solid substrate, we developed the direct synthesis of silane end-functionalized lipopolymers.[8] We used a poly(2-methyl- or 2-ethyl-2-oxazoline) backbone as the hydrophilic tether. The living character and the variability of the cationic ring-opening polymerization of 2-alkyl-2-oxazolines result in linear polymer chains,[9] with an adjustable degree of polymerization, low polydispersity, and quantitative end-functionalization at both ends of the polymer (Figure 2).[8] The hydrophilic/lipophilic balance (HLB) can be fine-tuned by the choice of the monomer, the degree of polymerization and the lipid head group. Using different degrees of polymerization for the hydrophilic tether, the intermediate polymer/water layer in the future model membranes might be adjustable.

To be able to vary the lipid head group, different lipids have been synthesized and used as initiators for the polymerization. This allows us to fine-tune the polymer architecture to construct stable and functional biomimetic membranes and to overcome the present limitations.

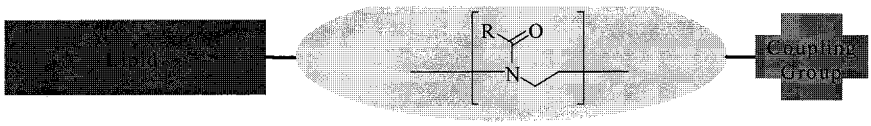
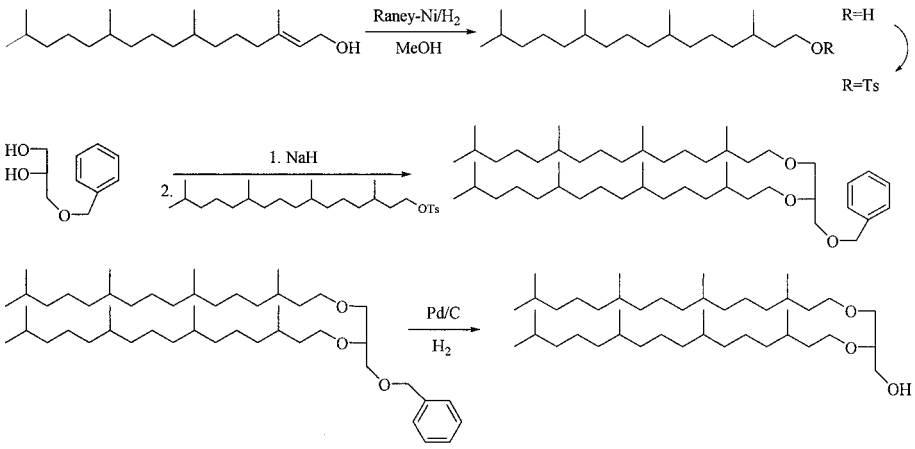


Figure 2. Cartoon of the 2-alkyl-2-oxazoline lipopolymer with a lipid head group, a hydrophilic polymer spacer and a silane coupling end function

Lipopolymer Synthesis

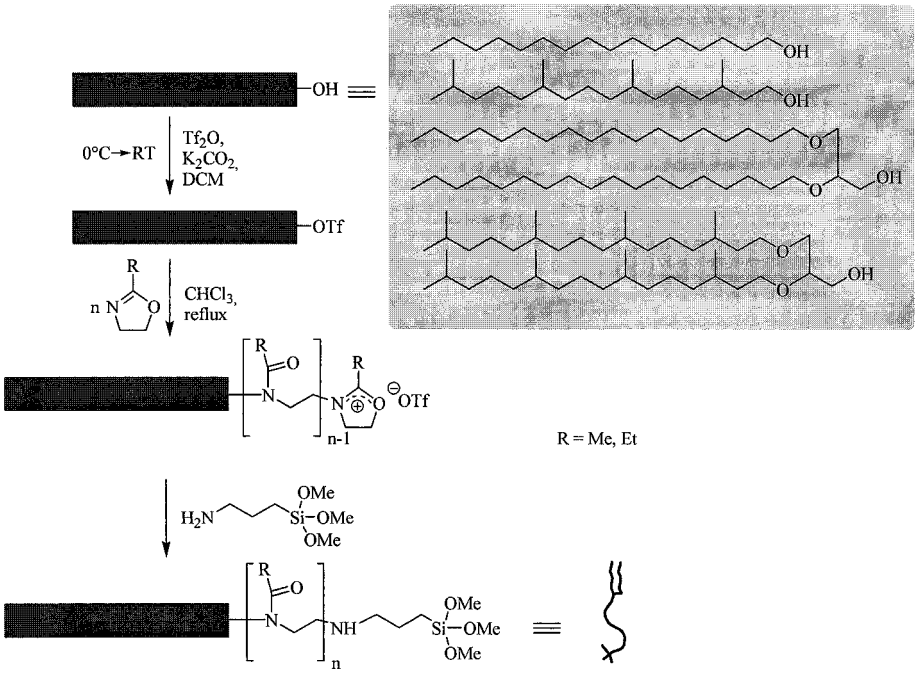
The potential variability of the lipopolymers structures and preservation of high control of the polymer architecture was the key criterion for the developed lipopolymer synthesis. The various lipid moieties were introduced into the polymer by the initiator method.[8] Alkyl moieties with a primary hydroxyl group were quantitatively converted into the corresponding triflates and then used as the initiator for the living cationic polymerization. This ensures a quantitative functionalization of the poly(2-oxazoline) with the lipid function. Most lipids are commercially available or can be readily synthesized. E.g. phytanol was obtained from phythol by reduction with Raney-Nickel.[10] The corresponding triflate was then directly used as the initiator or as a tosylate reacted with benzyl glycerol to yield, after cleavage of the benzyl protection group and triflatization the double chain analog lipid-initiator diphytanyl glycerol (Scheme 1).[11]



Scheme 1. Hydration of the phythol and synthesis of the diphytanyl glycerol lipid.

Other single and double chain lipids, such as *n*-hexadecyl triflate and 1,2-di-*n*-octadecyl-*s,n*-glyceroyl triflate were obtained directly from the alcohols. The choice of the lipids were motivated by (1) variation of the sterical need relative to the attached hydrophilic polymer to vary the critical packing parameter of the lipopolymer amphiphile, (2) matching of the alkyl chain length with the co-lipids in the future membrane construct, (3) good long-term stability (no ester bonds) and compatibility with the living polymerization.

The living cationic polymerization was then performed in chloroform to ensure good solubility of the initiator, the monomer and the lipopolymer during the entire polymerization. Each lipid was equipped with different polymer chain lengths (n=10, 20, 30, 40) simply by changing the initial initiator/monomer feed ratio for the living cationic polymerization. By this, a library of lipopolymers with different HLBs (HLB=hydrophilic/lipophilic balance) were obtained. The surface grafting function was finally introduced by a quantitative termination reaction using α-ω-amino alkylalkoxysilanes (Scheme 2).



Scheme 2. One-pot polymerization procedure resulting in end-functionalized lipopolymers.

The detailed procedures for the lipopolymer synthesis are published elsewhere.[8,12,13] The pure end-functionalized polymers can be obtained in good yields and narrow molecular weight distributions (see Table 1).

Table 1. Synthesized lipopolymers.

Polymer	Lipid	Monomer	$[M]_0/[I]_0$	PDI [1]
$C_{16}PEOx_{10}Si$	n-hexadecyl-	2-ethyl-2-oxazoline	10	1.17
$C_{16}PEOx_{20}Si$			20	1.33
$PhyPMOx_{10}Si$	phytanyl-	2-methyl-2-oxazoline	10	1.12
$PhyPMOx_{20}Si$			20	1.16
$PhyPMOx_{30}Si$			30	1.14
$2C_{18}PMOx_{10}Si$	1,2-di(*n*-octadecyl)-*sn*-glyceroyl-		10	1.04
$2C_{18}PMOx_{20}Si$			20	1.33
$2C_{18}PMOx_{40}Si$			40	1.42
$2C_{18}PEOx_{10}Si$		2-ethyl-2-oxazoline	10	1.16
$2C_{18}PEOx_{20}Si$			20	1.31
$2PhyPMOx_{10}Si$	1,2-di(phytanyl)-*sn*-glyceroyl-	2-methyl-2-oxazoline	10	1.04 [2]
$2PhyPMOx_{20}Si$			20	1.07 [2]
$2PhyPMOx_{40}Si$			40	1.16 [2]

[1] determined by GPC (solvent: chloroform, polystyrene standards)
[2] determined by GPC (solvent: dimethylacetamide, polymethylmethacrylate standards)

Lipopolymer monolayer

The first layer in a polymer tethered lipid membrane can either be prepared by a 'grafting from' [14] or a 'grafting onto' [15] process. Both procedures resulted in brush-like polymer monolayers with high grafting densities. Immediately after the grafting reaction the polymer layer was found to be homogenous and unstructured. A single submersion in water and consecutive exposure towards air at room temperature causes a self-organization of the lipopolymer layer into a hydrophilic water swollen polymer interlayer and an upper alkyl layer (Figure 3a).

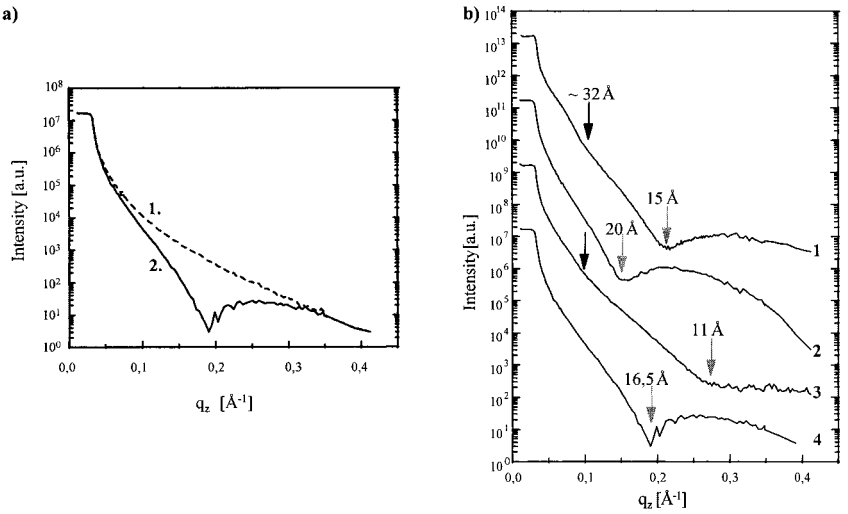


Figure 3. Specular x-ray reflection measurements of grafted lipopolymer monolayers on Si/SiO_2 wafers: **a) 1.** Unstructured lipopolymer monolayer of $C_{16}PEOx_{10}Si$, **2.** same sample after exposure to water. **b)** X-ray reflectivity curves for lipopolymer monolayers **1.** $2C_{18}PEOx_{20}Si$, **2.** $2C_{18}PEOx_{10}Si$, **3.** $C_{16}PEOx_{20}Si$, **4.** $C_{16}PEOx_{10}Si$. Bright arrows indicate minima corresponding to the alkyl layer, the dark arrow the position of the minima for the total layer thickness.

Depending on the critical packing parameters of the lipopolymer amphiphile, the thickness of the alkyl layer varies.[16] This can be explained by the difference in the cross-sectional area occupied by the polymer and the therefore given area for the alkyl part to tilt. In all cases, no crystallization of alkyl chains were observed in the polymer supported alkyl monolayer. Parallel x-ray reflectivity measurements of the bulk lipopolymers revealed for one candidate ($2C_{18}PEOx_{10}Si$) the spontaneous formation of double layers (layer spacing d = 6.4 nm). This multilayer formation could also be observed at substrate surfaces (Figure 4). Here, $2C_{18}PEOx_{10}Si$ was grafted onto a silicon wafer. However, the cleaning step to remove ungrafted polymer was not complete. Investigation of the surface by scanning probe microscopy (SPM; digital instrument, multimode, Nanoscope IIa controller, tapping mode) revealed a distinct terrace profile reflecting the height difference of single lipopolymer layers. The step height of ~3 nm for the single layer, as found by SPM, fits nicely to the layer thickness of a grafted lipopolymer layer of 3.2 nm as determined by x-ray reflectivity (Figure 3) and is about half of the found double layer spacing in bulk (6.4 nm).

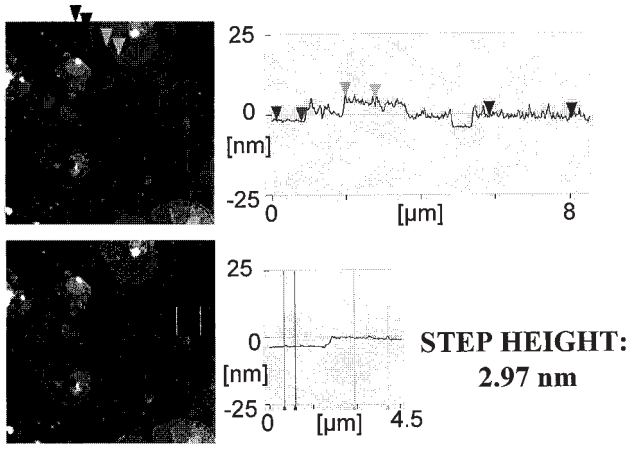


Figure 4. 10x10 μm^2 SPM scan (tapping mode) of a grafted $2C_{18}PEOx_{10}Si$ with topography.

Polymer Tethered Lipid Bilayers

For construction of the supported bilayer, air dried polymer monolayers prepared by the 'grafting from' or 'grafting onto' method were immersed to a solution of lipid vesicles in water. However, a formation of lipid bilayers could not be observed. Also numerous modifications of the procedure using different lipids and lipopolymers failed. Presumably, the exposure to water reorganizes the amphiphilic lipopolymer monolayer and a vesicle fusion did not occur. To build up stable polymer tethered lipid membranes, the procedure had to be modified in such a way that surface reconstruction of the first layer is suppressed. Regarding the mismatch in the cross-sectional area occupied by the polymer and the alkyl moiety, the alkyl layer has to be completed by additional lipids. This can be achieved by a step-wise preparation in which the first layer consisting of lipids and lipopolymers is pre-organized at the air/water interface of a Langmuir-Blodgett trough, compressed and then transferred onto the substrate. After an annealing step to complete the silanization reaction, a stable lipopolymer/lipid layer was obtained. Optimization of the LB-transfer procedure yielded homogenous and uniform layers for different lipopolymer/lipid compositions. This procedure allows us the deposition of pre-organized composite layers with defined grafting densities of the lipopolymer tether. The consecutive deposition of the upper lipid leaflet by fusion of lipid vesicles was successful.

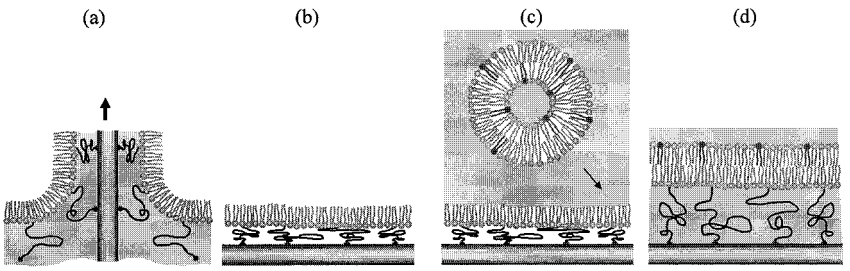


Figure 5. Schematic illustration of the stepwise preparation of a polymer-tethered membrane: (a) Langmuir-Blodgett (LB) transfer of a lipid / lipopolymer monolayer, (b) grafting of lipopolymers by annealing, and (c, d) spreading of the upper monolayer by vesicle fusion.

The additionally incorporated lipids in the first layer complete the outer hydrophobic layer and enable the vesicle fusion without the previously observed surface reconstitution in water.

The resulting polymer tethered lipid bilayers were investigated by fluorescence microscopy and their diffusion constants and mobile fractions by fluorescence recovery after photobleaching (FRAP). For these investigations, the stepwise construction procedure allowed the selective incorporation of fluorescent labeled lipids in the upper or lower layer. Inspection of the complete polymer supported bilayer showed a homogenous fluorescence for the labeled upper as well as the lower layer (Figure 6).

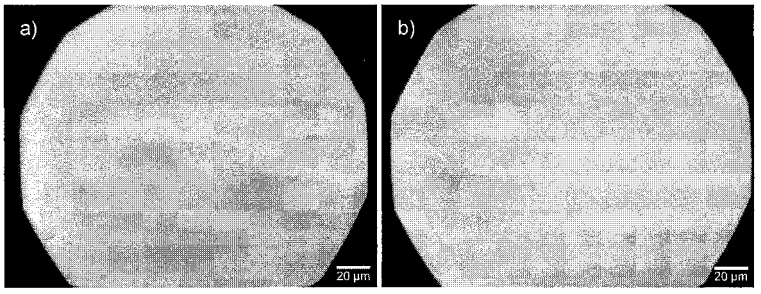


Figure 6. Homogenous polymer supported membrane with fluorescent labeled lipids in the a) lower and b) upper layer. (a) lower layer: 5 mol% $2C_{18}$PMOx20 / 94.8 mol% SOPC / 0.2 mol% Texas Red-PE; top layer: SOPC. b) lower layer: 5 mol% $2C_{18}$PMOx20 / 95 mol% SOPC; top layer 99.8 mol% SOPC / 0.2 mol% Texas Red-PE).

In continuous bleaching experiments, a good macroscopic diffusivity within both lipid layers was observed. Finally, FRAP measurements were performed to investigate the mobility and diffusion

constant of the lipids within both leaflets. In all cases, a high percentage (96-98%) of mobile lipids was observed. The diffusion constant, within the upper leaflet were found to be constant with D = 1-2 $\mu m^2 s^{-1}$. The diffusivity in the lower leaflet varied between 1.6 $\mu m^2 s^{-1}$ for low grafting densities of the lipopolymer (5 mol% $2C_{18}PMOx_{10}Si$) and 0.4 $\mu m^2 s^{-1}$ for high grafting densities (50 mol%). Long term stability tests by repeated FRAP measurements and inspection with fluorescence microscopy, performed with some samples, showed no significant change in appearance or lipid mobility. Such membrane constructs seem to keep their structural integrity over weeks.

The above mentioned results show the potential of the here presented method to construct polymer tethered lipid membranes with high structural integrity. Currently, detailed studies of the influence of different lipid head groups of the lipopolymers as well as various matrix lipids are carried out. Preliminary fluorescence interference contrast microscopy experiments showed a clear dependence of the substrate-membrane spacing from the degree of polymerization of the used lipopolymer tether. In fact, our preliminary experiments revealed that the membranes with long (n=40) polymer spacers can reside transmembrane proteins (platelet integrins) homogeneously, demonstrating the significant influence of the spacer length.[13]

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