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Hydrophilic Spacer Groups in Polymerizable Lipids: Formation of Biomembrane Models from Bulk Polymerized Lipids

R. Elbert, A. Laschewsky, and H. Ringsdorf

Abstract: A variety of polymerizable lipids containing a hydrophilic spacer group between the reactive group and the main amphiphilic structure have been synthesized. They were investigated in monolayers, liposomes, and multilayers. When the spacer concept was used, efficient decoupling of the motions of the polymeric chain and the amphiphilic side groups is achieved. Thus, the often found loss of the important fluid phases by polymerization is avoided. Polymeric monolayers of the spacer lipid, prepared either by polymerization in the monolayer or by spreading of prepolymerized lipid, exhibit nearly identical surface-pressure-area diagrams. Most distinctly, the successful decoupling of the motions of the polymer main chain and the membrane forming amphiphilic side groups is demonstrated by the self-organization of bulk polymerized spacer lipids to polymeric liposomes. In addition, spacer lipids are able to build polymeric Langmuir-Blodgett multilayers. The decoupling of the polymer main chain and the membrane-forming amphiphilic side groups enables the deposition of already polymeric monolayers onto supports to form defined multilayers. If, alternatively, monomeric monolayers are deposited and polymerized on the support, defects in the layers due to structural changes during the polymerization are avoided by the flexible spacer group.

Biomembrane Models and the Function of Spacers. The self-organization of amphiphiles in aqueous systems results in the formation of micellar, vesicular, and Langmuir-Blodgett (LB) multilayers. In general, these types of aggregates show poor stability compared to the biomembrane. The lack of stability can be overcome by the polymerization of reactive groups within the amphiphiles,1,2 but the resulting polymer chain interferes with the motion of the side chains and usually causes a decrease or even the loss of the fluid phases.3,4 More drastically, the reduced mobility due to the polymer backbone hinders efficient self-organization of polymerized lipids. Particularly, the formation of liposomes is prevented. Except for some hydrophilic copolymers with lipid side chains,5 polymeric liposomes were prepared only by polymerization of preformed monomer vesicles.6,7,23,24

Because effective biomembrane mimetic chemistry depends on the combination of both stability and mobility of the model membranes, several attempts have been reported in the literature to decrease the antagonism between polymer-enhanced stability and polymer-reduced mobility. Liposomes in a net8,9 polymeric liposomes were prepared only by polymerization of preformed monomer vesicles.6,7,23,24

An alternative concept is presented by the introduction of a hydrophilic spacer group between the main amphiphilic part and the reactive group of polymerizable lipids. The importance of spacer groups for the function of reactive centers fixed to carriers is well-known. Enzymes attached directly to polymers often loose their activity.13,14 A special spacer model has been discussed for polymeric drugs also.15 In addition the use of a spacer has been demonstrated for liquid crystalline side-chain polymers. They can be built systematically by the introduction of a spacer between the polymer backbone and the mesogenic side groups.15 Thus, the motion of the random polymer coil and the anisotropic mesogenic units is partially decoupled to allow the formation of liquid crystalline phases.

With that in mind, the use of the spacer concept for polymeric model membranes was investigated. In an attempt to decouple...
the motions of the polymeric chain and the amphiphilic side groups, hydrophobic spacer groups were introduced into polymeric lipids. The structure of such polymerizable lipids and

Scheme I. Principle of the Decoupling of the Polymer Main Chain Motion and the Self-Organization of the Amphiphilic Side Chains in Polymeric Spacer Lipids

the desired decoupling effect of the hydrophobic spacer group is illustrated in Scheme I. Chart I summarizes the spacer lipids synthesized. Cationic, nonionic, and anionic head groups were investigated.

Materials and Methods
Materials. Tetradecylfuran (THF), dichloromethane, and triethylamine (reagent grade) were liberated from water by percolation through a column of basic alumina (70–230 mesh; Merck). N,N,N-Trimethylethanol, carboxydimidazole, [1,2-dimethyl]pyridine, and succinic anhydride were obtained from Aldrich. Tetraoxtetraodecylphosphate (4).

The methacrylic ester was obtained from the alcohol by the same method as described for 1: yield, 0.35 g (32%); CH3OH/water mixture (5 g, 9.3 mmol) was reacted with succinic anhydride (1.39 g, 13.9 mmol) and 1.5 g of pyridine in chloroform at 55°C for 40 h. The reaction mixture was worked up with water, neutralized with aqueous sodium bicarbonate, and washed with water. After drying with magnesium sulfate and evaporation of the solvent, the reaction product was recrystallized from ether: yield, 5.3 g (89%); mp 52°C.

Sterification with tetracyclohexylmethyleneglycol was performed as described for 2: yield, 3 g (47%); mp 43–44°C.

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Sterification with tetracyclohexylmethyleneglycol was performed as described for 2: yield, 3 g (47%); mp 43–44°C.
Polymerization. Polymeric monolayers and liposomes were obtained by following methods: (a) The monomeric monolayers were flushed with nitrogen for 1 h and irradiated for 1 h with UV light. During the polymerization a surface pressure of 10 mN m\(^{-1}\) was kept. (b) 2,2'-Azoisobutyronitrile (0.02 mg) was added to an opaque suspension of the lipid which was then sonicated for 3–5 min and flushed with argon for 30 min. Polymerization of the liposomes was carried out at 60 °C for 20 h.

The lipids were polymerized in solution by the following method: To the lipid dissolved in toluene (10 mg/mL), 0.2 mg of 2,2'-azobisobutyronitrile was added. After the mixture was flushed with argon for 30 min, polymerization was carried out at 60 °C for 20 h. The polymeric lipid was precipitated with methanol and dissolved in CHCl\(_3\), for monolayer studies and spread. Liposomes were prepared from the precipitated polymer as described above.

Leakage. About 2 mg of the lipid was ultrasonicated in 2 mL of 0.1 M eosin in 0.1 M Tris/0.1 M NaOH. Free eosin was removed by passing 500 µL of the sonicated solution through a short column (13 X 180 mm) of Sephadex G-50 at 20 °C, with 0.1 M Tris-HCl/0.1 N KCl as eluent. The void volume fraction (\(V' = 7-10\) mL) contained the marked liposome. For determination of total entrapmed eosin, the dye was released from the vesicles by ultrasonication for 10 min and the fluorescence was measured.

Preparation of Multilayers. As support materials, polypropylene foils (Celgard 2400/Celanese, USA, and Trespaphen PED 6/Kalle, Federal Republic of Germany), poly(tetrafluoroethylene) (2-mm foil/Huth, Germany), polyester (Hostaphan RE 3,0/Kalle, Germany), and silanized quartz slides (Suprastil/Heraeus-Schott, Germany) were used. The flexible polymer sheets were attached to a Teflon sample holder.

Multilayers of 5 were built up by using subphases containing 1 g of CdCl\(_2\)-H\(_2\)O per liter. A surface pressure of 25 mN m\(^{-1}\) was applied at...
Figure 5. Surface pressure/area isotherms of the spacer lipid 2 on water (pH 5.5): $P_M$ = polymerized in monolayer; $P_s$ = prepolymerized in solution.

$20 \, ^\circ C$. The dipping speed was 5 cm/min downward and 2 cm/min upward.

The multilayers were polymerized at room temperature in air by a pen ray UV lamp Model 937-002 (Hamamatsu Corp.). The light intensity was 0.5 mW cm$^{-2}$; major intensity at 254 nm.

Results and Discussion

Spreading Behavior of the Monomers. Figures 1–4 show the spreading behavior of the monomeric spacer–lipids 1–4 at different temperatures. The surface pressure–area isotherms of all four lipids exhibit striking similarities. A liquid analogous phase is always observed, which stretches over a wide temperature range. The area occupied per molecule in the fluid phases are exceedingly large for all the lipids. This is caused by the large ethylene oxide containing spacer groups. At higher temperatures, only liquid analogous phases are observed. At lower temperatures, coexistence of liquid analogous and solid analogous phases is found. Whereas in the fluid phases large areas are occupied, the solid analogous phase is characterized by tight packing of the hydrophobic chains. Collapse areas down to 0.38 nm$^2$–molecule$^{-1}$ and high collapse pressures are found. This shows that the mobility of the spacer groups in the solid analogous state is reduced and cannot interfere with the packing of the chains, pointing to a partially stretched or helical orientation of the ethylene oxide spacer. Thus, the spreading behavior of the lipids 1–4 is dominated by the spacer groups in the fluid phases and by the packing of the hydrophobic chains in the solid analogous phases.

Spreading Behavior of the Polymers. Two pathways to ordered polymeric monolayers are possible. The usual method is the polymerization of monomeric monolayers. The alternative method, the spreading of polymerized lipids, usually leads to less ordered polymeric monolayers. Because the motions of the polymer chain interfere with the self-organization of the amphiphilic side groups, perfect packing is hindered.

In the case of the lipids 1–4 with hydrophilic spacers, the mobility of the amphiphilic groups is retained after the polymerization. This is exemplified by the surface pressure-area diagrams and the isobars of the lipids 2 and 4 in Figures 5–8. As shown, all the polymeric monolayers still exhibit fluid phases after the polymerization. Figures 6 and 8 show that the prepolymerized lipids have nearly the same spreading behavior as those lipids polymerized in the monolayer. This demonstrates that the spacer groups allow a polymer chain independent orientation of the amphiphilic side groups.

The isotherms in Figures 5 and 7 and the isobars in Figures 6 and 8 show some characteristic distinctions between monomers and polymers. The phase transition of the polymeric monolayers

MONOMERIC

High mobility of the spacer requires large areas in the fluid phase.

POLYMERIC

Reduced mobility of the spacer results in a decrease of the area occupied.

Figure 9. Schematic representation of the influence of the hydrophilic spacer on the area occupied by the lipids in the monolayer.

Figure 10. Freeze-fracture electron micrograph of liposomes from prepolymerized spacer lipid 4.

is shifted to higher temperatures. Of special interest is the fact that the areas occupied by the amphiphilic side groups of the polymer in the fluid phases are significantly lower than the areas occupied by the monomers, e.g., at 5 mN m⁻¹, 30 °C, for lipid 2, monomer, 1.35 nm²/molecule, polymer, 1.10 nm²/molecule. For lipid 4, monomer, 1.50 nm²/molecule, polymer, 0.95 nm²/molecule. This might be caused by the reduced independent mobility of the spacers after polymerization as shown schematically in Figure 9. However, in the solid analogous phase, the monomeric and polymeric monolayers show the same packing (Figure 7). In this case, the properties are controlled by the chain packing which is scarcely affected by the polymerization.

Formation of Liposomes. Of all biomembrane models, liposomes are the closest to cell membranes and therefore of a special interest. As discussed above, the stability of the low molecular weight liposomes is improved by the polymerization of reactive groups in the lipids.²⁻⁴,²⁻³,²⁻⁴ Similar to polymeric monolayers (Figure 5), there are two pathways of the formation of polymeric vesicles. The polymerization of preformed liposomes was first reported in 197⁹ and is now a widely used technique.¹ The alternative method is the formation of vesicles of prepolymerized lipids. Up to now, there is only one report about liposomes from polymers using hydrophilic copolymers with lipid side groups attached.⁵ It was interesting to see if the spacer concept, as discussed in the introduction, could lead to polymeric liposomes from prepolymerized lipids as shown in Scheme I.

Indeed, it could be shown that polymeric vesicles from the spacer lipids 1–4 can be produced by both methods, the polymerization of preformed vesicles and the ultrasonication of polymeric lipids prepolymerized in solution. In the latter case, the isolated polymeric lipids were ultrasonicated in water to yield opaque solutions. The formation of liposomes was shown by scanning electron microscopy and freeze-fracture electron microscopy (Figures 10 and 11).

Table 1. Phase Transition of Monomeric and Polymerized Liposomes Prepared from the Spacer Lipids 1–4 Determined by DSC

<table>
<thead>
<tr>
<th>Compound</th>
<th>T_C Monomer in K</th>
<th>T_C Polymer in K</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>315</td>
<td>322</td>
</tr>
<tr>
<td>2</td>
<td>290</td>
<td>305</td>
</tr>
<tr>
<td>3</td>
<td>306</td>
<td>322</td>
</tr>
<tr>
<td>4</td>
<td>282</td>
<td>323</td>
</tr>
</tbody>
</table>

Usually the polymerization of lipids in liposomes causes a loss of the phase transition,²⁵ comparable to the behavior of polymeric monolayers.²⁶ Typical for the polymeric vesicles prepared from spacer lipid monomers is the existence of a phase transition. The phase-transition temperature, T_C, is shifted to higher temperatures compared to the monomeric vesicles (Table I and Figure 12). Further, the transition seems to be narrowed.

This is in contrast to the rare phase transitions found in polymeared vesicles previously which were broadened and shifted to lower temperatures T_C. The results were explained by distortion

of the membranes due to the polymeric chains. In case of the polymerized vesicles prepared from the spacer lipids, the spacer groups decouple the motions of the polymeric chain and the self-organization of the amphiphile side chains. Hence, distortions of the membrane are avoided, and the enhanced cooperativity of the polymerized lipids leads to a narrowed phase transition shifted to higher temperatures $T_C$. Calorimetric investigations of polymeric vesicles derived from prepolymerized lipids could not be performed because the lipid content of vesicle dispersions is too low for measurements. In addition to electron microscopy, the formation of liposomes from prepolymerized lipids was shown by the entrapment of water-soluble dyes with the subsequent measurement of the leakage kinetics (Figure 13).

The leakage rates for polymeric vesicles prepared by both methods are comparable and are much lower than the leakage rates of the monomeric vesicles. Because the phase transition of polymerized vesicles is preserved, leakage rates of monomeric and polymeric vesicles in the fluid phase can be measured too. As seen in Figure 13, the leakage rate of the polymerized vesicles in the fluid phase at 60 °C is still much lower than the leakage rate of the monomeric vesicles at 20 °C. This demonstrates the reduced permeability of polymeric vesicles in the fluid phase also.

The comparison of the fluid phase and the gel phase shows that the entrapment of water-soluble dyes with the subsequent measurement of the leakage kinetics (Figure 13).

Table II. Thicknesses of Lipid Sandwich Bilayers in Multilayers, Determined by X-ray Wide-Angle Scattering

<table>
<thead>
<tr>
<th>Compound</th>
<th>Thickness of a Sandwich Bilayer in nm</th>
<th>Calculated Maximal Thickness of a Sandwich Bilayer$^a$ in nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>monomer</td>
<td>7.09</td>
<td>6.9</td>
</tr>
<tr>
<td>polymer</td>
<td>6.75</td>
<td>6.9</td>
</tr>
<tr>
<td>polymerized in the multilayer</td>
<td>5.68</td>
<td>7.3</td>
</tr>
<tr>
<td>polymerized in solution before spreading</td>
<td>5.68</td>
<td>7.3</td>
</tr>
<tr>
<td>polymer (red form)</td>
<td>5.68</td>
<td>7.3</td>
</tr>
</tbody>
</table>

$^a$ hydration of the head groups not considered

The spacer lipids for polymer multilayers prepared by either method was investigated.

The polymerization of built-up multilayers has been investigated for several years. The covalent linkage of the amphiphiles within the layers enhances the stability of the multilayers. However, normally the polyreaction causes disturbances in the layers or even formation of defects. This can be demonstrated by the example of the hexacosa-10,12-diyanoic acid, a typical polymerizable lipid used for LB multilayers. The comparison of the measured thickness of a sandwich bilayer of 5.68 nm (see Table II) and the calculated value for the maximal stretched conformation of about 7.3 nm indicates a tilting of the lipids in the layers. During the polymerization, the tilting is reduced and the thickness of the bilayers enlarged to 6.15 nm. This structural change disturbs the order of the multilayer as can be seen by the decrease of the number of orders observed in X-ray scattering experiments. In bilayers, polymerization can even cause defects (see Figure 14).

These problems might be overcome by the use of lipids containing the hydrophilic spacers. The separation of the polymer chain and the membrane-forming part of the molecule should minimize the disturbing influence of the polymer backbone. Based on these considerations, we investigated the possibility to build up LB multilayers with lipid 4. In fact, we succeeded to produce nearly defect-free multilayers, as electron micrographs show (see Figure 15). Monolayer transfer takes place during the downward and the upward dips of the support (Y-type deposition). It is interesting that the large spacer group does not interfere with the deposition process. The surface pressure–area isotherms (Figure 4) suggest that the spacer group must be oriented to a high degree in the solid analogous phase, extending into the subphase. This orientation might enhance the transfer of the monolayers. The built-up multilayers can be readily polymerized by UV light. Figures 16 and 17 show the polymerization behavior of multilayers of 4, as followed by UV spectroscopy. In contrast to topochromatically controlled reactions, complete conversion can be achieved.

The electron micrographs in Figure 15 show that no defects caused by the polymerization can be observed even after complete conversion of the monomer. This fits well with the data obtained by X-ray experiments. Wide angle scattering experiments show that the thickness of the sandwich bilayers is only slightly reduced by polymerization from 7.09 to 6.95 nm (Table II). How the spacer groups are arranged in the multilayers is not clear. The

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measured thickness of a sandwich bilayer of 7.09 nm lies between the maximal stretched conformation of the amphiphile of about 9.9 nm and the maximal stretched length of the membrane-forming part (up to the phosphate group) of 6.0 nm (Table II). A possible explanation might be the tilting of the lipids which would mean a tilting angle of 45° against the layer plane. Alternatively, a nonstretched conformation of the spacer may be considered. For example, an U-turn in the middle of the spacer would reduce the spacing to about 7.5 nm, which is nearly the value found. Also a helical structure of the ethylene glycol can be discussed. A nonstretched conformation could also explain the hydrophilic surface of the multilayer during the upward dip. In the case of the fully stretched conformation, the methacrylate group would be exposed at the surface and, thus, a hydrophobic surface would be expected.

The second method to obtain polymeric multilayers, the transfer of polymeric monolayers onto solid supports, should void the problems connected with the polyreaction in the multilayer. Further, a larger variety of polyreactions and monomers can be used, especially if the polymeric monolayers are prepared by the spreading of prepolymerized amphiphiles.

However, the transfer of polymeric monolayers has been scarcely used up to now because the polymeric chain within the membrane-forming part of the compounds leads to severe problems with deposition. Normally, using the LB technique, only incomplete transfer to the support is achieved. Additionally, due to the strongly reduced flexibility of the monolayers, the transfer causes defects in the layers. Often, no more defined layer structure is found.

Because of these problems, to date, the technique of Langmuir and Schaefer has been used to build multilayers from polymeric monolayers. But, this method is restricted to coat small supports, and hence the applications of such polymeric multilayers are limited.

Because of the efficient decoupling of the motion of the polymer main chain and the self-organization of the amphiphilic side groups found in polymeric monolayers and polymeric vesicles, we investigated the use of prepolymerized spacer lipids for the preparation of LB multilayers. For that purpose, polymeric lipid was spread (see Figure 8) and the surface pressure adjusted at 30 mN m⁻¹ corresponding to the condensed phase of the monolayer. Applying slow dipping speeds downward and upward, the polymeric layers can be transferred onto various support materials as silanized quartz, polypropylene, poly(tetrafluoroethylene), or polyester. Regular Y-type deposition of the monolayers is observed. The thickness of the sandwich bilayer was determined by X-ray wide angle scattering experiments (Table II). As can be seen, the value corresponds to the thickness of multilayers of polymerized on the support. The considerations about the arrangement of the lipid side chains in the multilayer are the same as pointed out above for the multilayers prepared from monomeric lipid. The good agreement of the thicknesses of the polymeric
multilayers built by the different methods suggests that the structure of the layers is independent of the preparation technique chosen. But, without additional investigations including such of other spacer lipid systems, the result should not be overestimated.

Conclusions

The spacer concept, that means the partial decoupling of the motions of the polymer backbone and the orientable, anisotropic side groups, proved to be useful for polymerizable lipids. The main problem of biomembrane models, that the stability achieved by polymerization will exclude the important mobility of the membrane, can thus be overcome. Polymeric model membranes of lipids with hydrophilic spacers still show the desired fluid phases. Moreover, the flexibility of polymerized lipids is sufficient to make possible monolayer spreading and vesicle formation of polymerized lipids. These unique properties offer wide possibilities in biomimetic systems. For example, proteins or enzymes can be incorporated in liposomes efficiently now, which before suffered either from poor stability in case of monomeric lipids or from reduced fluid phases and damage by the polymerization process in case of polymerized vesicles. The multilayer systems, which are of special technical interest, also profit by the spacer concept. Distortions caused by the polymerization process are minimized, and thus the high quality of the layers is preserved. Furthermore, the flexibility of the polymeric lipids due to the spacer group allows one to build up multilayers from polymeric monolayers. Thus, potential distortions of the lipids created by the polyreaction are avoided, and the scope of polyreactions suited to polymerize amphiphiles is enlarged considerably.

Registry No. 1, 96326-70-4; 1 (alcohol), 96326-75-9; 2, 96326-71-5; 2 (alcohol), 96326-76-0; 3, 96326-72-6; 3 (alcohol), 96326-78-2; 4, 96326-73-7; 4 (phosphoric acid dichloride), 74123-29-8; 4 (dialcohol phosphate), 96326-79-3; 4 (hydrolysis product), 96326-80-6; [2,3-bis(hexadecanoyloxy)propyl]dimethylamine, 96326-74-8; triethyleneglycol monoiodohydrin, 62573-16-4; methacrylic acid, 79-41-4; N,N-dioctadecylsuccinamide, 37519-63-4; tetraethyleneglycol, 112-60-7; 1,2-O-dihexadecylglycerol, 6076-35-3; 2,3-bis(hexadecyloxy)propyl succinate, 96326-77-1; phosphorus oxychloride, 10025-87-3.