Monatshefte für Chemie 135, 13–21 (2004)
DOI 10.1007/s00706-003-0101-6

Monatshefte für Chemie
Chemical Monthly
Printed in Austria

Domain Formation in Lipid Bilayer Membranes: Control of Membrane Nanostructure by Molecular Architecture

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Received August 13, 2003; accepted August 19, 2003
Published online November 27, 2003 © Springer-Verlag 2003

Summary. Lipid-polymer conjugates with differing hydrophobic to hydrophilic dimensions were prepared. The polymer part consisted of hydrophilic poly(2-methyl-1,3-oxazolines) (*POZO*), whose chain length can be controlled by living polymerization methods. The lipid-polymer conjugates were incorporated into vesicles composed of *L*-α-dimyristoylphosphatidylcholine (*DMPC*) or *L*-α-dipalmitoylphosphatidylcholine (*DPPC*) in amounts of up to 25 mol%. The formation of domains within the lipid bilayer membrane is observed by atomic force microscopy (AFM), thermal analysis (DSC), and fluorescence spectroscopy. The synthetic design of the lipids allows control over the distribution of polymer chains on the vesicle surface.

Keywords. Lipid bilayer membrane; Domains; Lipid-polymer conjugate; Fluorescence; Atomic force microscopy.

Introduction

Lipid bilayer membranes offer a wideness of structural variability, which is exemplified by the formation of cells, vesicles, and other types of lipid aggregates. Besides this macroscopic structure formation, mixtures of different lipids within a lipid bilayer membrane can cluster into domains on the surface of vesicular aggregates [1]. Sizes of these domains may vary between several nanometers up to many microns in diameter [2]. This structure formation is of enormous biological significance (named "rafts" or alternatively "domains") and influences a variety of biological processes such as cholesterol transport [3], viral entry [4], and protein targeting [5]. As reviewed recently [1] a variety of structural effects can be hold responsible for the formation of domains in lipid membranes. A special pair of

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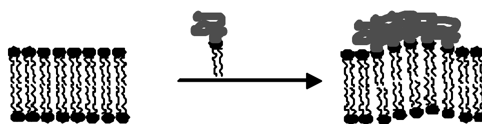


Fig. 1. Domain formation by incorporation of lipid-polymer conjugates into lipid-bilayer membranes

two, three, or more lipids may phase separate laterally under domain formation due to (a) mismatch of lipid chain length, (b) entirely differing head groups, (c) a special intermolecular interaction between cholesterol and sphingolipids, (d) the adsorption of macromolecules onto the surface of lipid vesicles, as well as (e) phenomena induced by Ca^{2+}-ions acting on bilayers composed of phosphatidylserines.

We are interested in the influence of hydrophobic chain length and hydrophilic head group interactions on the domain formation in lipid bilayer membranes (Fig. 1). Lipid-conjugates can be incorporated into membranes in order to impart favorable properties onto the membrane surfaces [6]. By choice of the appropriate lipid-conjugates this leads to higher membrane stiffness as well as increased circulation times of vesicles in the human body when applied to the living organism due to steric shielding of the vesicular surface [7].

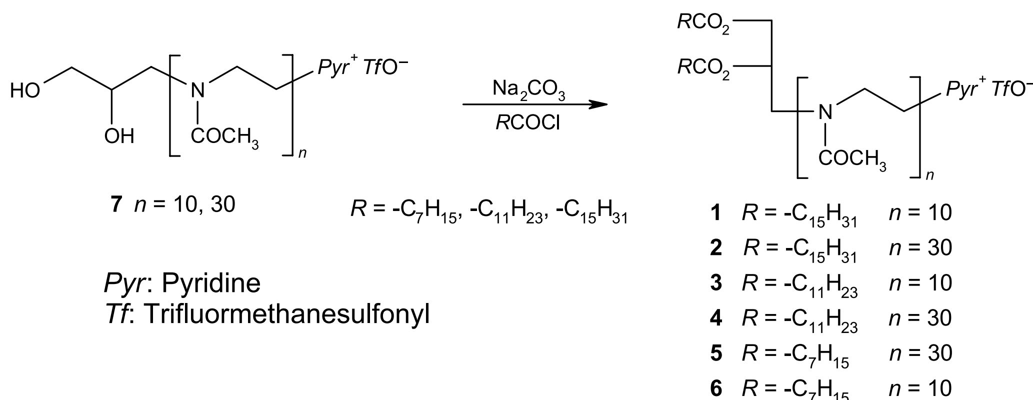
Usually hydrophilic polymers such as poly(ethyleneglycols) and poly(acylethyleneimines) are used as the hydrophilic part in the lipid-conjugate for this purpose [8]. Important for the stabilization process is the equal distribution of the lipid-polymer-conjugate on the surface of the vesicles. Since usually about 10 mol% of lipid-polymer conjugate are incorporated into the bilayer membrane for obtaining the stabilization effect, the formation of domains consisting either of lipid-polymer conjugate and islands of the surrounding lipid is likely. It can be anticipated, that – depending on the hydrophilic/hydrophobic portion within the lipid-polymer-conjugate – domain formation will be observed on the vesicle surface. The present account deals with the incorporation of lipid-polymer conjugates into vesicles composed of *L*-α-dimyristoylphosphatidylcholine (*DMPC*) or *L*-α-dipalmitoylphosphatidylcholine (*DPPC*) and the investigation of their domain formation by DSC-, AFM-, and fluorescence measurements.

Results and Discussions

Synthesis of the Lipid-Polymer Conjugates

The preparation of the lipid-polymer conjugates is described in Scheme 1 and follows a procedure developed in our laboratory [9]. Briefly a deprotection/acylation strategy was employed starting from the diacylglycerol-telechelic polymers **7** (with varying chain lengths $n = 10$, 30), which had been prepared via a combination of the polymerization-reaction of 2-methyl-1,3-oxazoline using functional triflic acid initiators [9].

The critical step was the acylation of the diol **7** using an extremely high excess of alkanoic acid chloride which proved necessary for obtaining complete acylation of both hydroxyl moieties. Solid dry sodium carbonate emerged as the base of



Scheme 1

Table 1. Molecular weight data of the lipid-polymer conjugates **1–6**

Lipid	$M_{w\,(\mathrm{GPC})}$	M_w/M_n	$M_{n\,(\mathrm{th})}$
1	1700	1.06	1615
2	3400	1.04	3315
3	1500	1.04	1503
4	3100	1.08	3203
5	3000	1.03	3121
6	1300	1.02	1421

choice in terms of acylation efficiency and nonaqueous workup conditions. The resulting lipid-polyoxazoline conjugates **1**–**6** are obtained with defined molecular weights (as judged by GPC-analysis) and low polydispersities obtained by gel permeation chromatography (Table 1). Both the theoretical and experimental molecular weights show good correlation combined with polydispersities (M_w/M_n) below 1.1.

Incorporation of the Lipid-Conjugates into Vesicles

In a first round the lipid-polymer conjugates were incorporated into vesicles. To this purpose lipid mixtures were prepared by coevaporation with the basic lipid forming the vesicle (either *DMPC* or *DPPC*) and the lipid-polymer conjugates **1**–**6** in amounts between 0–25 mol%. The vesicles were then formed by hydration of a lipid film and subsequent extrusion at a temperature above the main phase transition temperature, yielding multilamellar vesicles with a broad size distribution. Lipid **6** obviously was too hydrophilic to form vesicles and was thus not considered for further analysis.

Analysis by High Sensitivity DSC

A first round of investigations was dedicated to ultrasensitive DSC-measurement. Table 2 shows the composition of the vesicles together with their phase transition

Table 2. DSC-data of the lipid-conjugates in *DPPC* (*DMPC*)-vesicles

Entry	Lipid	mol%	Lipid[(a)]	$T_{(\text{main-trans})}/°\text{C}$	$T_{(\text{pre-trans})}/°\text{C}$
1	**1**	10	*DPPC*	39.8	28.5
2	**1**	15	*DPPC*	39.0	27.1
3	**1**	20	*DPPC*	37.6/39.2	–
4	**1**	20	*DMPC*	17.0–30.0	6.3
5	**2**	10	*DPPC*	40.0	30.1
6	**2**	15	*DPPC*	38.8	27.1/34.2
7	**3**	10	*DPPC*	37.2/40.0	28.1/30.5
8	**3**	24	*DPPC*	35–42	–
9	**3**	20	*DMPC*	10–25	–
10	**4**	10	*DPPC*	39.3	–
11	**5**	10	*DPPC*	39.6	32.2/30.1

[(a)] Pure *DPPC*: $T_{(\text{main-trans})} = 41.6°\text{C}$, $T_{(\text{pre-trans})} = 34.2°\text{C}$; pure *DMPC*: $T_{(\text{main-trans})} = 24.1°\text{C}$, $T_{(\text{pre-trans})} = 14.0°\text{C}$

behavior. It is well known, that upon observing the main phase transition in lipids, domain formation can be proven [10]. It has been shown, that the liquid/crystalline to fluid transition is strongly affected by the phase state of the lipids in a lipid mixture within the bilayer membrane. Domain formation can be proven by splitting of the main transition.

All lipid conjugates **1**–**5** incorporated in *DPPC* led to a slight depression of the main transition of the parent lipid *DPPC* at amounts of up to 10 mol% of the lipid conjugates **1**–**5** as seen in entries 1, 2, 5, 6, 7, 11, and 10. However, there was a large shift in the pretransition temperature even at low concentration of the incorporated lipid. Thus, temperature shifts of up to 6°C were already observed at concentrations of 10 mol% (entry 1, 2, and 11). When using higher amounts of up to 24 mol% of lipid-polyoxazoline conjugate peak splitting of the main phase transition was observed (Fig. 2), which is indicative of domain formation at temperatures below the main phase transition of pure *DPPC*.

There was, however, a strong dependence on lipid structure and concentration: Whereas lipid **1** as a 10% mixture in *DPPC* yielded only a broadening of the main transition, a splitting effect was already present at 10 mol% of lipid **3** in *DPPC*. For lipid **1** a concentration of 20 mol% of lipid in *DPPC* was necessary to yield a splitting of the main transition, whereas 10 mol% of lipid **5** in *DPPC* yielded only a broadened main transition centered at 39.6°C. The pretransition temperatures in general were moved to lower temperatures with increasing amount of lipids **1**–**5** and were not detectable at concentrations above 20 mol%. Similar trends were observed in mixture with *DMPC* (entry 4 and 9).

In order to probe whether the hydrophilic headgroup is influencial in domain formation lipids **1** and **2** as well as lipids **3** and **4** were compared, which differ only in the length of their respective hydrophilic headgroups: There was a significant change in the pretransition temperatures (compare entry 2 to 6 and entry 7 to 10) but no significant difference was observed in the main transition temperatures. This hints at a major contribution of the hydrophilic headgroup to hydrational effects

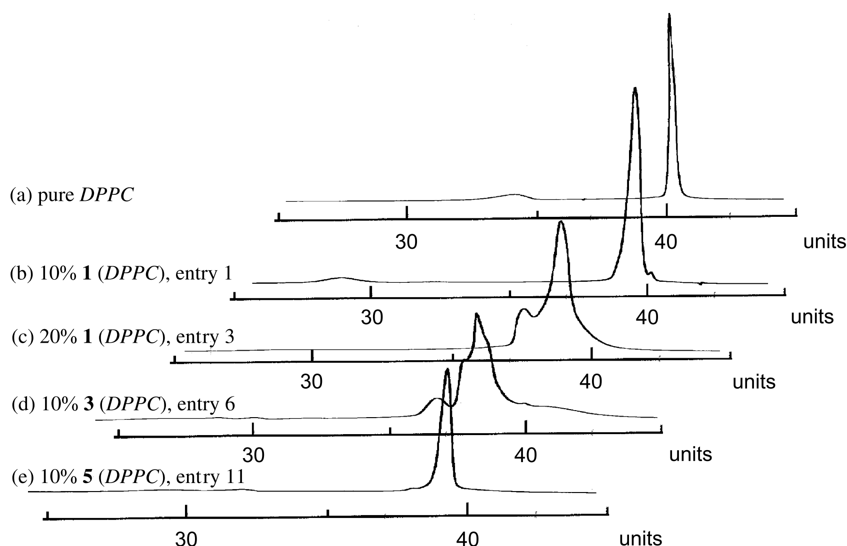


Fig. 2. High sensitivity DSC of lipid-conjugates in vesicles composed of *DPPC*

present in the formation of the "ripples" during pretransition and only a minor contribution on domain formation effects.

AFM-Measurements

Besides DSC-methods we were interested in search for other methods to prove the domain formation in lipid membranes. Domains can be also visualized by atomic force microscopy (AFM) [11]. This method is able to detect height differences after a vesicle has adsorbed onto a surface and rolled up into a lipid bilayer membrane of the surface of mica. We have investigated this with vesicles composed of pure *DPPC* and those composed with a mixture of 24 mol% of lipid **3** in *DPPC* (entry 8 in Table 2). The corresponding AFM-pictures are shown in Fig. 3.

Pure *DPPC* as a monolayer on mica shows height differences of 4.5 nm indicative of the height-variations of a pure lipid bilayer membrane. The lipid mixture (24 mol% of lipid **3** in *DPPC*, Fig. 3b) clearly shows additional height differences of $\sim$1 nm. The width of the height differences varies from several 10 nm to about 200 nm, indicating plaques of this size within the planar, spread lipid bilayer membranes. This can be interpreted as domains, from which the polymers (due to their extended conformation) are sticking out. The height variations of about 1 nm are suggestive of extended brushes of the hydrophilic polymers and support this view.

Fluorescence Measurements

Fluorescence anisotropy is a valuable tool to probe the mobility of lipids in bilayer membranes. Since the presence of domains consisting of pure *DMPC* and domains consisting mainly of lipid **3** is proven by DSC-measurements, we have used the adsorption of a hydrophilic dye (coumarine 6) to probe the fluorescence anisotropy

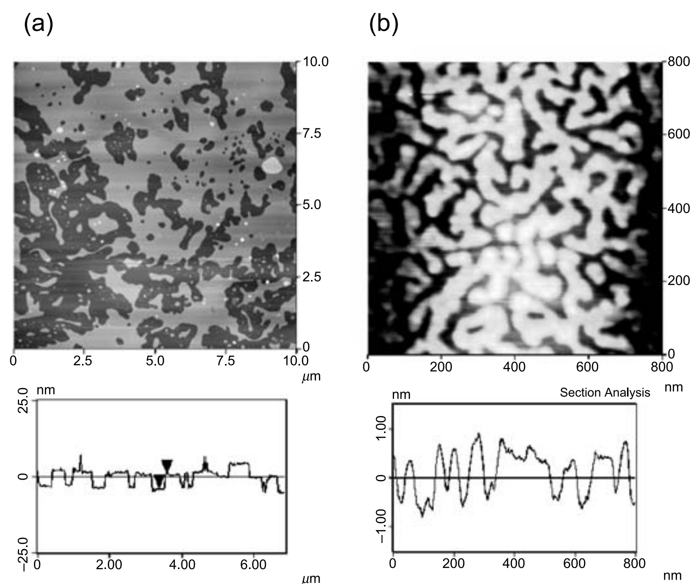


Fig. 3. AFM-pictures of (a) pure *DPPC*-bilayer membranes, (b) mixture of lipid-polymer conjugate **3** (24 mol%) in *DPPC*

of the vesicular membranes. Coumarine 6 is known to enrich within the hydrophilic portion of the membrane (*i.e.*, the hydrophilic headgroup moiety) as proven by its solvatochromic shift. Figure 4 shows the anisotropy of fluorescence with increasing temperature of vesicles composed with different mixtures of lipid **3** in *DMPC* (entry 9 in Table 2).

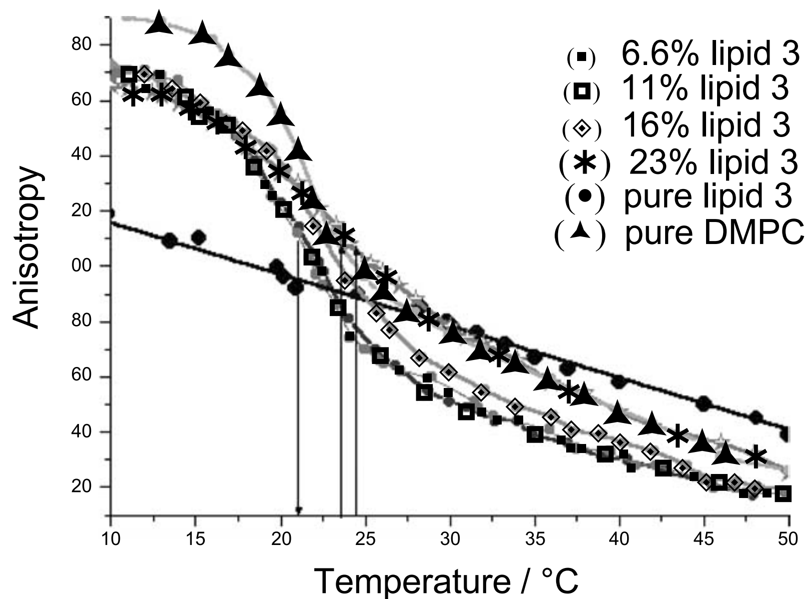


Fig. 4. Temperature dependency of the fluorescence anisotropy of various mixtures between lipid-polymer conjugate **3** and *DMPC*

Clearly, there is a drastic change of properties between 20–25°C due to the thermotropic gel/liquid transition of *DPMC*. Thus, the most rigid environment of coumarine 6 is represented by the *DMPC* bilayer. By addition of lipid **3** the headgroup region of the bilayer is less compact, thus allowing more movements of the coumarine 6 molecules. Therefore the anisotropy of fluorescence is lowered by addition of **3** in both the fluid and the solid phase of the bilayer. Interestingly, the curves are scattering for the fluid phase and not for the solid phase. This can be interpreted in that the coumarine 6 is shifted to the lipid **3** solid phase domains being much more flexible in the headgroup region than the rather compact *DMPC* headgroup region. Controversely it seems that for very high lipid **3** concentrations (23 mol% of lipid **3**) coumarine 6 is located in the *DMPC* rafts which is documented by the rise in the degree of anisotropy of fluorescence to exactly the same values as for pure *DMPC* only. Thus the coumarine 6-molecules are expelled to the domains of lipids **3** in the solid phase of the bilayer membrane.

Conclusion

We have demonstrated the formation of domains in mixtures of lipid-polymer conjugates with *DPPC* or *DMPC* in lipid bilayer membranes inside of vesicles. Three methods (DSC, AFM, and fluorescence) prove the domain forming process. The hydrophobic portion of the lipid-polymer conjugates was found to be the determining element leading to domain formation rather than the hydrophilic polymer chain. The domains show a size range in the 10–200 nm range when applied as a bilayer film on mica, which is in accordance with previous measurements in literature. The defined domain formation in relation to the molecular architecture of the molecules offers the possibility to "engineer" domains in lipid membranes. Experiments concerning this process are in progress.

Experimental

Materials and Methods

Solvents were dried and distilled, if not otherwise indicated. Reagents were purchased from Sigma-Aldrich and used without further purification. The synthesis of the precursors (diacyl-glycerol terminated lipid-polyoxazoline conjugates **7**) was described in Ref. [9]. Lipids (*L*-α-dimyristoylphosphatidylcholine (*DMPC*) and *L*-α-dipalmitoylphosphatidylcholine (*DPPC*) with purities $>99.8\%$ were purchased from Sigma Aldrich. NMR-spectroscopy was measured on a Bruker AC-400 instrument operating at 400 MHz for (^{1}H) nuclei and 100.16 MHz for (^{13}C) nuclei. Fluorescence polarization measurements were performed with a Perkin-Elmer LS 50-B spectrofluorimeter equipped with a thermostated cell holder. Vesicles for fluorescence spectroscopy were about 60 nm in diameter.

Preparation of Vesicles

Multilammelar vesicles were prepared as 5 m*M* solutions of lipid content in phosphate buffer (*PBS*, *pH* = 7.4, 10 m*M* phosphate, 150 m*M* NaCl) by hydrating lipid films. Thus, the appropriate amount of lipid (*i.e.*, 6.6 mg of *DPPC*) was weighed into a glass vial and dissolved in 1 cm^3 of a 1:1 mixture of

methanol:chloroform. The solvent was removed by passing a stream of nitrogen over the mixture until complete dryness was achieved. The vial was then filled with argon and three glass beads with a diameter of 2 mm together with 2 cm^3 of warm *PBS* buffer (60°C) were added. The flask was vortexed for 30 s and then incubated at 60°C for 30 min yielding a 5 m*M* solution of multilammelar vesicles (MLVs).

DSC-Measurements

DSC samples were degassed for 30 min before filling into the DSC-tube. In order to obtain equilibrium spectra samples were measured *via* two scans separated by an equilibration time. The scan rate was 10 min per h. The usual scan range started at 4°C and ran up to 60°C. Spectra obtained in two subsequent measurements proved reproducible.

AFM-Measurements

AFM-measurements were conducted on a Nanoscope III of Digital Instruments Inc. Samples were deposited on the mica surface by dipping the freshly cleaved mica plate into a diluted solution (approx. 5 m*M* in lipid concentration) for about 9 h, after which the solution was rinsed with deionized water. The dipping time is important in achieving appropriate thickness of only one layer of the bilayer membrane on the mica surface. Subsequent measurements were then conducted with silicon tips in the tapping mode using frequencies between 300–400 kHz.

General Procedure for the Preparation of the Lipid-Polyoxazoline Conjugates **1–6**

2.12 g of dry sodium carbonate and 1.00 g of the diacylglycerol-terminal polymer **7** [9] were disperged in 20 cm^3 of dry chloroform. The calculated amount of the acid chloride (20 mmol) was added dropwise and the mixture was stirred for 18 h at 20°C. The solid components were then removed by filtration and the filtrate was evaporated *in vacuo*. The residue was dissolved in 150 cm^3 of acetonitrile and extracted twice with 100 cm^3 of *n*-hexane. In order to achieve a final purification (complete removal of excessive acid chloride) the acetonitrile phase was purified by continuous liquid/liquid extraction with 400 cm^3 of *n*-hexane. The acetonitrile phase was then collected, evaporated to dryness and dried in high vacuum to yield the lipid-polymer conjugates **1–6** as slightly yellow, highly hygroscopic solids. The yield of the final product was in all cases between 95–97%. (Since all compounds have a homologous structure, only one representative ^{1}H, ^{13}C, and FT-IR spectrum is given. The index *p* denotes the resonances of the repetitive methylene $(CH_2)_n$ unit of the fatty acid chain. The index "*n*" denotes the length of the poly(oxazoline) chain. ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.70$ (t, 6H, $COCH_2CH_2(CH_2)_pC\underline{H}_3$), 1.10 (m, {4*p*}H, $COCH_2CH_2(C\underline{H}_2)_pCH_3$), 1.40 (m, 4H, $COCH_2C\underline{H}_2(CH_2)_pCH_3$), 2.00 (m, {3*n*}H, $COCH_3$), 2.20 (m, 4H, $COC\underline{H}_2CH_2(CH_2)_pCH_3$), 3.35 (m, {4*n*-4}H, $NC\underline{H}_2C\underline{H}_2N$), 3.90 (m, 2H, $NC\underline{H}_2CH_2N^+$), 5.00 (m, 2H, $NCH_2C\underline{H}_2N^+$), 7.90 (m, 2H, *Pyr*H), 8.40 (m, 1H, *Pyr*H), 9.00 (m, 2H, *Pyr*H) ppm; ^{13}C NMR ($CDCl_3$, 10 MHz): $\delta = 14.50$ ($COCH_2(CH_2)_p\underline{C}H_3$), 21.10 ($CO\underline{C}H_3$), 21.50–33.00 ($COCH_2(\underline{C}H_2)_pCH_3$), 34.50 ($CO\underline{C}H_2(CH_2)_pCH_3$), 42.00–50.00 ($N\underline{C}H_2\underline{C}H_2N$), 59.70 ($OCH_2CH\underline{C}H_2N$), 63.30 ($O\underline{C}H_2CHCH_2N$), 68.00 ($OCH_2\underline{C}HCH_2N$), 120.00 (q, $J = 320$ Hz, CF_3), 128.60 (C_{Pyr}), 145.90 (C_{Pyr}), 146.00 (C_{Pyr}), 174.00–175.00 ($\underline{C}OCH_3$, $\underline{C}OCH_2(CH_2)_pCH_3$) ppm; FT-IR (KBr): $\bar{\nu} = 2927$ (m, CH_2), 2856 (m, CH_2), 1743 (m, OC=O), 1637 (s, NC=O), 1484, 1422, 1261, 1160, 1032 cm^{-1}.

Acknowledgment

We are grateful to the Austrian Science Fund (FWF) for financial support *via* the projects FWF 14844 CHE and 13294 CHE.

References

[1] Binder WH, Barragan V, Menger FM (2003) Angew Chem, in press
[2] Anderson RGW, Jacobson K (2002) Science **296**: 1821
[3] Simons K, Ikonen E (1997) Nature 569
[4] Raulin J (2002) Progr Lipid Res **41**: 27
[5] Melkonian KA, Ostermeyer AG, Chen JZ, Roth MG, Brown DA (1999) J Biol Chem **274**: 3910
[6] Lasic DD, Needham D (1995) Chem Rev **95**: 2601
[7] Lasic DD, Martin F (1995) Stealth Liposomes. CRC-Press, Boca Raton
[8] Zalipsky S, Hansen CB, Oaks JM, Allen TM (1996) J Pharm Sci **85**: 133
[9] Einzmann M, Binder WH (2001) J Polym Sci A: Polymer Chemistry **181**: 57
[10] Silvius JR, del Guidice D, Lafleur M (1996) Biochemistry **35**: 15198
[11] Milhiet PE, Giocondi MC, Le Grimellex C (2002) J Biol Chem **277**: 875