Radical Polymerization of Amphiphiles in a Two-Dimensional Solution (Mixed Vesicles)†

H. Meier, I. Sprenger, M. Bärmann, and E. Sackmann*

Physik Department E22 (Biophysics Laboratories), Technische Universität München, James-Franck-Strasse, 85748 Garching, Germany

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ABSTRACT: We studied the size distribution of macromolecular amphiphiles dissolved in a lipid bilayer matrix of phospholipids, which were polymerized photochemically or by thermal decomposition of a radical-forming initiator. The degree of polymerization, N, was measured by gel permeation chromatography using chloroform/methanol (93/7, v/v) as solvent. The reliability of the method was checked by comparison with a transesterified polymer, electron microscopy, and quasi-elastic light scattering. Evidence is provided that polymerization yields larger chains in two-dimensional than in three-dimensional solutions. The photochemically induced polymerization yields the largest polymers ($N \approx 10^4$) by short-time irradiation (≤5 min at 208 nm), which is just long enough to produce one or two radicals per vesicle. Longer irradiation times lead to photodecomposition of the polymers, and after 10 min, only oligomers of $N \leq 4$ remain. High sample turbidities and multilamellar vesicles require longer times due to light scattering. Polymerization mediated by thermal decomposition of a water soluble initiator at 73 °C yields sharp distributions of long chain polymers ($N \approx 10^4$) by adjusting the initiator to amphiphile ratio in such a way that polymerization occurs within a reasonable time (≈1 h). With increasing initiator concentration, the size distribution is shifted to smaller chains ($N \approx 300$) due to simultaneous initiation of several chains per vesicle. Large polymers ($N \geq 10^4$) are formed (both photochemically and by initiator decomposition) by both methods for amphiphile concentrations as small as 25 mol %. From the finding of a threshold concentration above which large polymers are formed, the lifetime of the radical at the growing end is estimated as $t_r \approx 1-3 \times 10^{-7}$s. Formation of large polymers leads to lateral phase separation of macrolipids. The resulting domain structure of the vesicle is unstable, leading to budding and subsequent detachment of small vesicles which may be essentially composed of only one large macrolipid. The budding could be a consequence of a strong spontaneous curvature of the polymerized domains caused by a sterically induced contraction of the head groups with respect to the chains.

Introduction

Partially polymerized membranes are generally prepared by radical polymerization of polymerizable amphiphiles in fluid bilayer vesicles or monolayers. The process is initiated photochemically or by thermal decomposition of initiator. Mixing of the amphiphiles with nonpolymerizable lipids provides many advantages:[1-4] large vesicles are easier to prepare; two-dimensional macromolecular solutions can be prepared for studies of fundamental properties (such as scaling laws) of such low dimensional complex fluids;[3] in potential applications for the encapsulation of reactants or drugs, such partially polymerized vesicles can be more easily manipulated—such as for the transient opening and closing of pores.[5]

Despite 10 years of research in this field, the question of the degrees of polymerization which can be achieved by photochemical polymerization in two-dimensional solutions (monolayers and vesicles) is still open. In the case of amphiphiles carrying diacetylene groups in each hydrocarbon chain, large cross-linked polymers are formed by topochemical polymerization.[1,5,6] In the case of linearly polymerized amphiphiles with the molecules interconnected via functional groups attached to the head groups of the amphiphiles, the published estimates of the polymerization degree, N, range from $N \approx 2-4$ to $N \approx 2000$. Measurements of N of a linearly polymerized amphiphile[6] were performed by gel permeation chromatography (GPC) after transesterification. N was found to be of the order of 2000.[6] For the amphiphile (4,16)-POMECY (shown in Figure 1), which was polymerized photochemically in vesicles of dimyristoylphosphatidylcholine (DMPC), a value of $N \approx 300$ has been estimated by lateral diffusion measurements combined with Monte-Carlo simulations.[7,8]

Here, we present the results of our systematic study of the polymer size of (4,14)-POMECY and (4,16)-POMECY (**I**), polymerized in mixed DMPC/POMECY vesicles either photochemically or by thermal decomposition of the water soluble initiator ACVA (**II**). N was measured by GPC as a function of (i) the concentration of the polymerizable amphiphile in the DMPC bilayers, (ii) the intensity of the irradiating light and the time of irradiation, (iii) the concentration of initiator, and (iv) the relaxation time of the vesicle suspension after sonification. Absolute values of the degrees of polymerization, N, of the polymerized lipids were determined by comparison with poly(methyl methacrylate) standards (PMMA). In order to verify that this procedure yields reliable degrees of polymerization, we compared the values of N for one (or several) sample prior to and after transesterification resulting in a polymer chain without attached lipids. For further verification of the polymer sizes N determined by GPC of the amphiphile, absolute values of N were also measured by quasielastic light scattering and electron microscopy. Previous results[8] that very large polymers ($N \approx 10^4$) can indeed be formed photochemically in DMPC vesicles, provided the irradiation time is short enough to avoid photochemical decomposition of the polymers, were verified. We found that large polymers of $N > 10^4$ can always be prepared via thermal decomposition of initiator by appropriate adjustment of the initiator/POMECY ratio. A detailed study of the variation of the polymer size distribution with the concentration of the amphiphile in DMPC vesicles was performed, and a threshold

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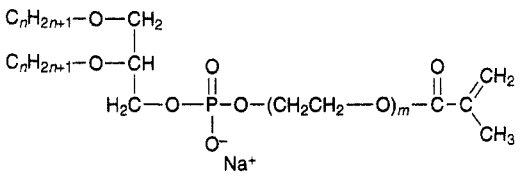
concentration was found from which the lifetime of the radicals at the growing end of chains was estimated.

Freeze fracture electron microscopy and phase contrast microscopy studies of the vesicle stability after polymerization showed that embedding the amphiphiles in DMPC vesicles does not prevent the destabilization of giant vesicles. Domains were formed within the vesicle membrane and subsequently detached in the form of small vesicles by budding and fission. We infer that these may consist of single polymer chains saturated with monomers.

Materials and Methods

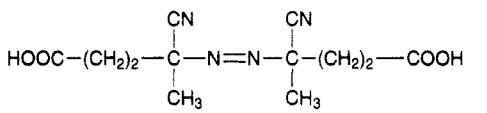
Materials. Dimyristoylphosphatidylcholine was a commercial product (Sigma). The polymerizable amphiphiles (4,-14)-POMECY (with myristoyl chains) and (4,16)-POMECY (with hexadecyloxy chains) (**I**) were gifts of the Ringsdorf



(I)

1,2-*O*-diacylglycero-3-[2 [2'-(methacryloyloxy)tris(ethyleneoxy)]ethyl] phosphoric acid diester sodium ($m = 4$; $n = 14$ and 16)

group. They were chemically pure, as shown by thin-layer chromatography. The water soluble initiator 4,4'-azobis[4-cyanovaleric acid] (ACVA) (**II**) was also a gift of the Ringsdorf group.



(II)

4,4'-azobis[4-cyanovaleric acid] (ACVA)

Vesicle Preparation. Vesicles were prepared as follows: A thin film of the lipid mixture was deposited on the wall of a glass flask by the usual solvent evaporation technique[1] (3/1 chloroform/methanol). Millipore water was then added (2 mg of lipid/mL of water), and the lipid was swollen for 1 h at some degrees above the chain melting transition temperature of the lipid with the highest melting point. Liposomes were formed by mechanical agitation of the glass flask, and small unilamellar (or thin-walled) vesicles were formed by sonification. In order to study the effect of the vesicle size and lamellarity on the photochemical polymerization, large vesicles were prepared by variation of the sonification time from 0 to 5 min.

Polymerization Procedures. The photochemical polymerization was performed with a low-pressure 24 W deuterium lamp from a UV/vis spectrometer. The lamp emits from 190 to 775 nm. The vesicle suspension was contained in a 2 mm quartz cuvette. In order to study the photopolymerization as a function of irradiation intensity, the distance between the cuvette and the lamp was varied. At the surface of the quartz bulb, the light flux was calculated to be 50 mW cm^{-2}. In the present work we report only results obtained for two distances ($d = 22$ mm (~10 mW cm^{-2}) and $d = 33$ mm (~4.6 mW cm^{-2})).

The initiator-induced polymerization was performed by adding ACVA to the aqueous solution used for the swelling of the vesicles. The dispersion was kept at 73 °C just long enough to ensure polymerization.

Transesterification of Polymer. The polymerized amphiphiles were precipitated in methanol. Sodium methylate was added in high excess at a mole ratio of sodium methylate: POMECY monomer of 1:10, and the reaction mixture was refluxed at 80 °C for 24 h. The success of the transesterification was controlled by three independent tests: (1) determination of liberated phosphate by complex formation with molybdenum, (2) observation of additional peaks on the HPLC chromatogram, and (3) comparison of the NMR spectra of transesterified polymer, PMMA, POMECY monomer, and

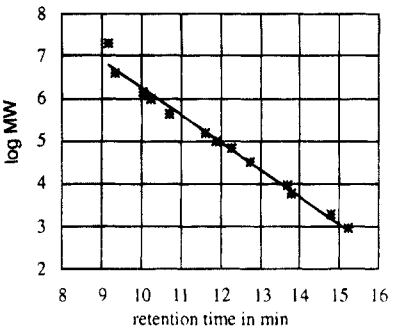


Figure 1. Calibration of GPC HPLC system used for our experiment using PMMA standards. The eluent was chloroform/methanol (93%/7%), the flow rate was 1 mL/min, and the column consisted of poly(styrene) cross-linked with divinylbenzene.

POMECY polymer. The transesterification product of the polymer backbone is identical in chemical composition with PMMA.

HPLC and GPC—Chromatography. A Gynkotek HPLC pump equipped with a mass detector (Zinsser, Frankfurt/M, FRG), a column oven (Merck, Darmstadt, FRG), a solvent degasser (Gynkotek, Germering, FRG), and an integrator (Shimatsu, Düsseldorf, FRG) was used. For the separation, the size exclusion principle was applied. The column material was poly(styrene) cross-linked with divinyl benzene. Two columns (purchased from Merck) with pore sizes of 5 and 10 nm, corresponding to exclusion limits of 10^2 and 10^7 Da, respectively, were combined. With pure chloroform as mobile solvent, the polymerized amphiphile strongly adsorbed to the column material thus delaying the retention time by hours. This problem was overcome by addition of 7% methanol to the eluent. The flow rate was typically 1 mL/min.

Special care was taken to calibrate the column for the relative molecular mass determination (in terms of hydrodynamic radii). In principle such a calibration should be performed with the same type of polymer, the same solvent, and under identical conditions (pH, temperature, running time). Since standards of polymerized amphiphiles are not available, we employed the following procedure.

We first calibrated the column with poly(methyl methacrylate) (PMMA). As shown in Figure 1, the logarithm of the molecular weight decreases linearly with retention time, t_R. The lower limit of resolution ($t_R \approx 9$ min) corresponds to a molecular mass of $M = 10^7$ Da. According to Elias, a large number of polymers other than PMMA lie on the same straight line[9,10] showing that the PMMA standard can be used for a large class of polymers. In order to clarify whether the calibration curve of Figure 1 could be used also for the polymerized amphiphile, we performed the following tests:

(1) One part of an isolated fraction of the polyamphiphile was transesterified, and its retention time, t_R, was compared with that of the nontreated part. The primary structure of the transesterified polymeric amphiphile was expected to be identical with the primary structure of commercial PMMA, whereas its molecular mass corresponded to only about 15% of the molecular mass of the (nontransesterified) polymerized amphiphile at the same degree of polymerization. Nevertheless it has been found that the retention times of the polymer amphiphile and the transesterified polymer agree remarkably well. Therefore all further experiments were performed with nontransesterified polymer. The values of the relative molecular mass, M_R, of polymerized POMECY as determined by GPC with PMMA as reference standard must be multiplied by a factor of 7 in order to get the correct molecular mass.

(2) As a second test, the hydrodynamic radii of the polymer amphiphiles were measured by diffusion measurements using quasi-elastic light scattering (QELS). For that purpose the polymer amphiphile was dissolved in pure chloroform. The hydrodynamic radius was obtained from the diffusion coefficient using the Stokes–Einstein equation and assuming that the amphiphile assumes a random coil.

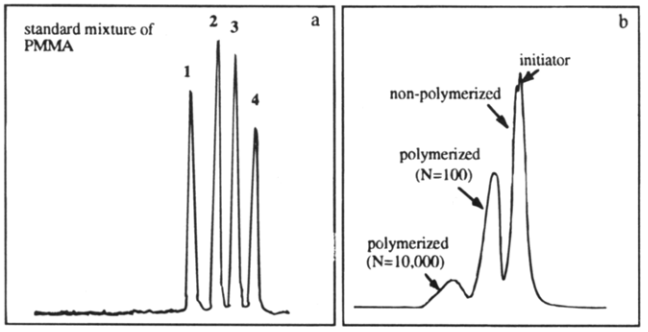


Figure 2. (a) Peaks of the calibration with PMMA: (1) 1.2×10^6 Da, (2) 152 000 Da, (3) 33 500 Da, and (4) 6000 Da. (b) Example of a chromatogram of nontransesterified (4,16)-POMECY polymerized photochemically. The POMECY to DMPC molecular ratio was 70/30.

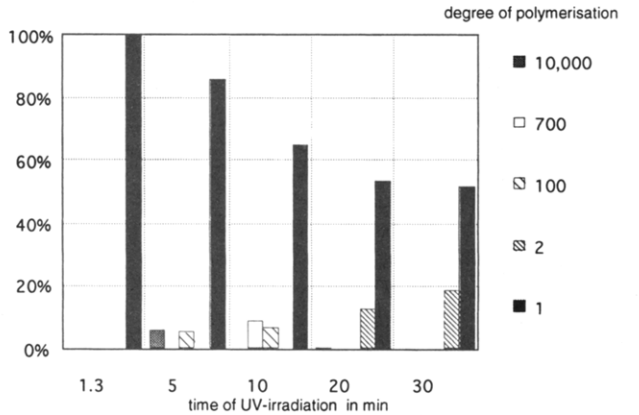


Figure 3. Variation of polymer yield with UV irradiation time for 1:1 (4,14)-POMECY: DMPC mixtures. The bars represent the percentage of monomer and oligomer. The degree of polymerization is divided into groups of $N > 10\,000$, $N \approx 700$, $N \approx 100$, and $N \approx 2$ as indicated on the right side. Note that monomer also includes DMPC of the equimolar mixture.

(3) A third test was based on electron microscopic observation of the various GPC tractions of nontransesterified polymerized amphiphiles. These are well soluble in chloroform, forming most probably inverted micelles with the hydrocarbon chains pointing toward the outside. A small droplet of a differently diluted chloroform solution of the fraction studied was deposited on mica. After evaporation of the solvent, the sample was contrasted with platinum which was deposited by the rotary shadowing technique under an oblique angle of 10°. The replicae were observed with a Philips EM 400 electron microscope.

Experimental Results

Photochemical Polymerization. The degree of polymerization, N, was determined as a function of (i) the time of irradiation, (ii) the composition of the DMPC–POMECY mixture, (iii) the irradiation light intensity, and (iv) the structure and lamellarity of the vesicles.

Consider first the effect of the variation of the time and intensity of irradiation. Figure 3 shows a histogram of the polymer size distribution obtained for a 1:1 (4,-14)-POMECY:DMPC mixture after 1.3, 5, 10, 20, and 30 min of irradiation. At $t_{irr} \leq 1.3$ min, no appreciable polymerization was observed. After 5 min, 28% of (4,-14)-POMECY was polymerized and 50% of the macrolipid exhibits degrees of polymerization $N > 10\,000$. After 10 min, the turnover has increased to 70% but the maximum degree of polymerization is $N \approx 700$. After 20 min, 93% are turned over but only small oligomers are found (together with DMPC and lyso products).

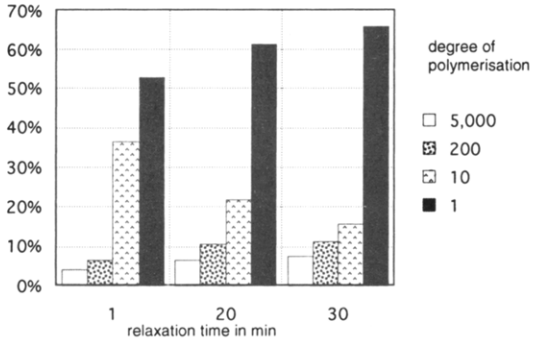


Figure 4. Dependence of polymer yield in 50:50 (4,14)-POMECY:DMPC mixtures on the time of annealing after sonification of vesicle suspension prior to polymerization. Bars give the percentages of monomer and oligomer of the average size indicated at the right side of the histogram.

After 2 h, only dimers and many lyso products are found (besides DMPC).

Similar results were found for other mixtures of DMPC and (4,14)-POMECY (containing 25% and 75% of the POMECY). The only difference was that the time of irradiation required to obtain degrees of polymerization $N > 10\,000$ was somewhat longer (ca. 2.5 min). Since the same holds for nonsonificated dispersions, we attribute this effect to the higher turbidity of the samples. Light is scattered by the liposomes residing behind the front wall of the cuvette, and it takes longer to form radicals in the center and rear parts of the cuvette. This turbidity effect also provides an explanation for previous observations that photopolymerization yields large polymers if single-shelled giant vesicles are irradiated under a microscope while only short chains are obtained for turbid vesicle suspensions.[7]

The intensity of the irradiating light has a drastic effect on polymer turnover and size distribution. With increasing intensity, the turnover increases sharply. As one example we studied a 1:1 mixture of (4,14)-POMECY and DMPC. Variation of the distance between the lamp and the cuvette from 23 to 33 mm (corresponding to a decrease in intensity by factor of 2) decreased the turnover from 34% to 9% at an irradiation time of $t_{irr} = 5$ min. The maximum polymer size increased from $N \approx 900$ to $N \approx 2000$.

The size of the vesicles and the packing of lipids in the vesicles have a remarkably strong effect on the turnover and size distribution of the polymer. As shown in Figure 4, the relaxation time between sonification and polymerization affects the formation of large polymers as follows: the fraction of the largest polymer was smaller after 1 min annealing time than after annealing for 20 or 30 min. This type of relaxation effect is partly attributed to the healing out of the very high densities of packing defects in highly curved vesicles. Another possible explanation is vesicle fusion. Evidence is provided below that for small vesicles the maximum size of the polymer is determined by the number of POMECY molecules in one monolayer.

Initiator-Induced Polymerization. The radical-induced polymerization by thermal decomposition of the initiator became effective only above 70 °C. No appreciable polymerization was found at temperatures up to 60 °C, even after 5 h of incubation. At $T > 70$ °C, the polymer yield was >80% after 0.5 h. The half-time of the initiator decomposition, which is 7.4 h at 73 °C, is 20 h at 60 °C and is expected to yield a measurable quantity of radicals after 5 h of incubation at 60 °C. Therefore, the lack of significant polymerization is

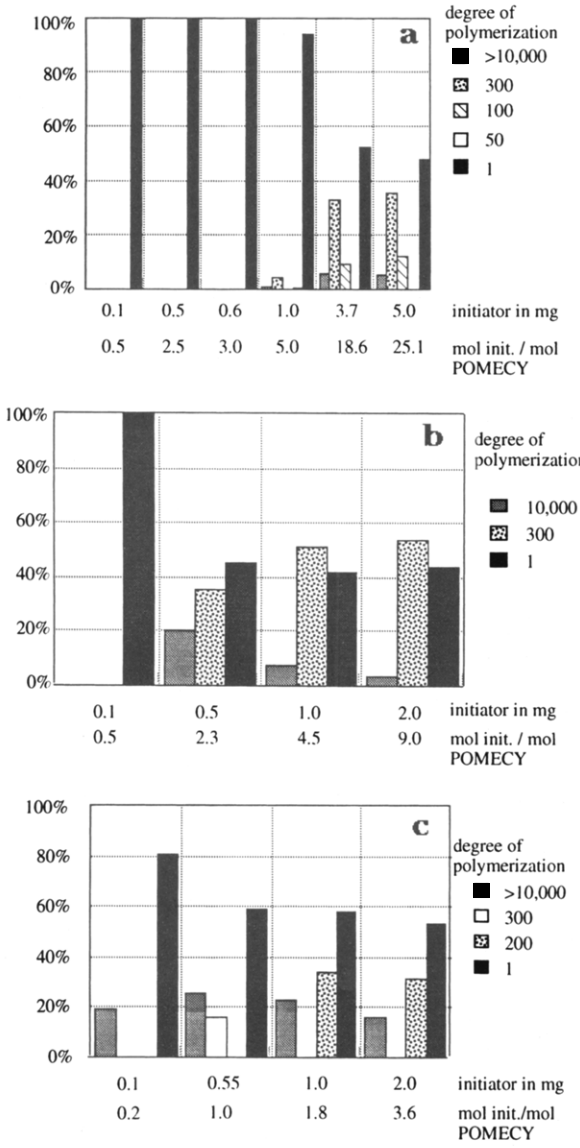


Figure 5. Dependence of polymer yield on initiator concentration in 70:30 POMECY:DMPC mixtures. Total lipid concentration was 0.4 mg/mL (a), 1.0 mg/mL (b), and 2.5 mg/mL (c). Bars give the percentages of monomer and oligomer of the average size indicated on the right side. The total amount of initiator and the initiator-to-POMECY molecular ratio, m, are indicated below the histogram. Note that monomer fraction also includes DMPC. The polymerization time was 1 h, and the reaction volume was 2 mL in each case.

attributed to the insolubility of the initiator below 70 °C but not to decomposition kinetics.

The initiator is well soluble after addition of NaOH. However, since this led to salt precipitation after disolution of the vesicle suspension in chloroform, this procedure was avoided. The effective initiator concentration may thus be smaller than the concentration of the ACVA added.

In order to keep the reaction temperature as low as possible, polymerization was performed at 73 °C in the present study. The polymer size distribution was studied as a function of (i) the total lipid concentration, (ii) the initiator-to-POMECY mole ratio, m, and (iii) the reaction time. Figure 5 shows how the polymer yield in mixed vesicles containing 70% (4,16)-POMECY varies with the total lipid concentration and the initiator-to-(4,16)-POMECY mole ratio.

The smaller the total lipid concentration, the larger the initiator-to-polymerizable lipid mole ratio, m, must

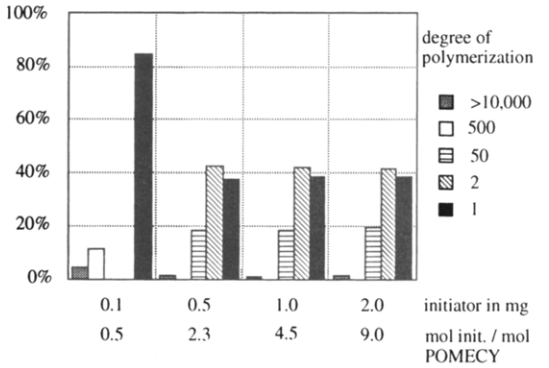


Figure 6. Yield of (1,14)-POMECY polymerized in dioxane at various initiator concentrations. Reaction conditions and data grouping are as in Figure 5. The total lipid concentration was 1 mg/mL.

be in order to obtain appreciable yields of high molecular weight polymers. Thus, for 0.4 mg/mL total lipid, m must be larger than $m = 1$ in order to obtain large polymers ($N > 10^4$; Figure 5a), and for 1 mg/mL, it must be larger than $m = 0.5$ (Figure 5b), while for 2.5 mg/mL, $m = 0.1$ is sufficient to obtain large yields of polymers with $N > 10^4$ (Figure 5c).

One observes a remarkable decrease in the relative yield of large polymers with increasing initiator concentration, while the fraction of small polymers increases correspondingly. For a total lipid concentration of 1.0 mg/mL, the relative yield of the fraction with $N > 3000$ decreases from 20% to 3% when m increases from $m = 0.5$ to $m = 2.0$, whereas the yield of the fraction with $N \approx 200$ increases from 35% to 55%.

For comparison, the initiator-triggered polymerization of (4,16)-POMECY was also studied in an organic solvent, dioxane. The results are presented in Figure 6. Again the relative yield of four fractions with $N > 10\,000$, $N \approx 500$, $N \approx 50$, and $N \approx 2$ was determined as a function of the initiator-to-(4,16)-POMECY mole ratio. A substantial amount of the large polymer fraction ($N > 10^4$) was found only for the smallest initiator concentration ($m = 0.1$), while for higher values of m, the bulk of the (4,16)-POMECY yielded only smaller polymers ($N \leq 500$). (4,16)-POMECY was completely turned over after 1 h, provided $m > 0.5$. Comparison of Figures 5 and 6 strongly suggests that the yield of high molecular weight polymers is much higher in the two-dimensional solution than in dioxane.

Effect of Reaction Time. In order to observe possible effects of prolonged exhibition of the polymerized vesicles to ACVA radicals, the polymer distribution was analyzed as a function of reaction time. For a 70:30 (4,16)-POMECY:DMPC mixture polymerized at 73 °C in the presence of an initiator-to-POMECY mole ratio of 3.34, we found that after a reaction time of 0.5 h about 80% of polymerizable lipid had been turned over into high molecular weight macrolipid with $N \geq 10^4$ (data not shown). This yield is increased to ≈90% after 1.5 h. It does not change after more prolonged exposure to radicals up to 3 h. Since the half-time of ACVA decomposition is $t_h = 7.5$ h, we conclude that the polymer is stable with respect to thermally produced radicals.

The existence of a minimum value of m is a consequence of the finite average lifetime of the initiator radicals, I*. These can decay by recombination with other initiator radicals or react with water and the walls of the reaction flask before reaching the vesicle surface.

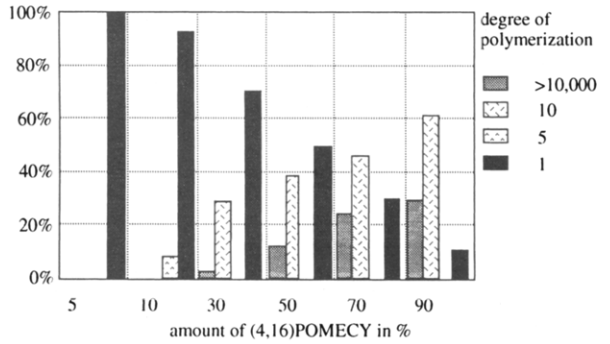


Figure 7. Variation of size distribution of polymerized (1,-14)-POMECY with concentration of monomer in DMPC vesicle. Polymerization was performed by ACVA decomposition at 73 °C. The initiator-to-POMECY mole ratio was $m = 0.99$; the incubation time was 1 h. The relative amounts are again given in terms of percentage of total lipid including DMPC.

In fact, one can roughly estimate the radical lifetime, as will be shown in the Discussion section.

Variation of Polymer Size Distribution with Bilayer Composition. Experiments were performed on photochemically and thermally induced polymers. Figure 7 shows some results for the latter case. An appreciable yield of the high molecular weight polymer ($N > 10^4$) was obtained already above a threshold (4,-16)-POMECY concentration of 25%. The relative amount of polymer was 2% at 30% POMECY. It increased to 9% at 50% and to 33% at 90% (4,16)-POMECY. A further remarkable result was that the relative amount of small oligomers ($N \geq 10$) increased drastically by increasing the POMECY content of the vesicles.

In the case of photopolymerization under optimized conditions (7.5 min irradiation), the bilayer composition influenced the polymer size distribution similarly. However, the total yield of polymers was much smaller than in the case of the thermally induced polymerization (data not shown).

Determination of Polymer Size by Electron Microscopy. In order to check whether the relative polymer sizes obtained by GPC were reliable, some fractions were analyzed by electron microscopy. For this purpose the isolated fractions were dissolved in chloroform and replicae for electron micrographs were prepared as described above. Figure 8 shows transmission electron micrographs for three different polymer fractions. Comparison of micrographs 3 and 4, respectively, shows that the average volume increases by a factor of 100 if the degree of polymerization rises from $N = 300$ to $N = 10\,000$. Assuming that chloroform is a reasonably good solvent and the polymer radius scales roughly as $\sqrt{N}$, one expects a volume ratio of ~200, in reasonable agreement with the observation. This provides at least some further evidence of the reliability of the determination of the degree of polymerization by GPC using nontransesterified polymer.

Phase Separation and Stability of Vesicles. A rough phase diagram of the DMPC/(4,16)-POMECY mixture has been established previously.[7] Before polymerization the mixtures of lecithins and (4,16)-POMECY are miscible. This is expected for two reasons: (i) For the same chain lengths, the phase transition temperatures of POMECY are only a few degrees below the values of monomeric lecithin and nearly ideal behavior is expected. (ii) (4,16)-POMECY is charged, and the repulsive electrostatic pressure favors ideality of the mixture.[7,11]

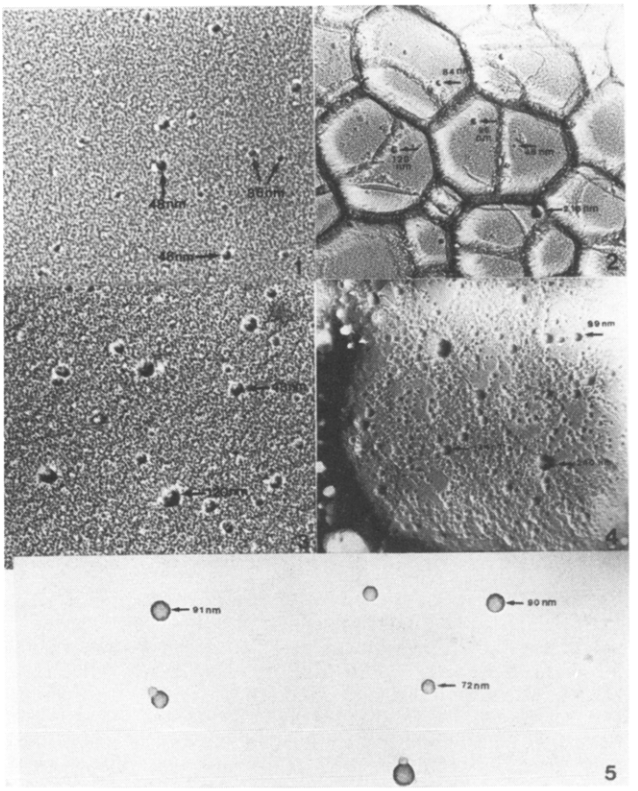


Figure 8. Determination of polymer size by electron microscopy. Observation of polymerized fractions dissolved in chloroform. (1) GPC fraction at retention time 11 min, corresponding to $N = 300$ (average radius, $\langle r \rangle \approx 40$ nm). (2–4) GPC fraction at retention time 8 min, corresponding to $N = 10\,000$ (average radius, $\langle r \rangle \approx 200$ nm). (5) GPC fraction at retention time 9 min, corresponding to $N = 5000$ (average radius, $\langle r \rangle \approx 80$ nm).

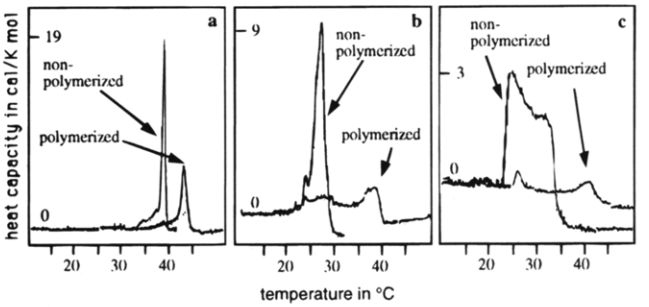


Figure 9. Differential scanning calorimetry of mixtures of (4,16)-POMECY with phosphatidylcholines before and after polymerization. (a) Plot of heat capacity as function of temperature for 75:25 mixture of (4,16)-POMECY and DPPC before and after polymerization. (b) Same as in plot a for 72:25 mixture of (4,16)-POMECY and DMPC. (c) Same calorimetric run as in plot a for pure (4,16)-POMECY.

A remarkable finding is that for equimolar POMECY/DMPC mixtures the vesicles become small even after only slight mechanical agitation. They exhibit a rather narrow size distribution with an average diameter of 500 nm. This is most probably a consequence of the negative charge and the large head group of the polymerizable amphiphile, since an equimolar mixture is expected to allow optimal packing. After polymerization the situation is very complex. Figure 9 shows calorimetric curves from DSC measurements for the 75:25 mixtures of (4,16)-POMECY with DPPC (Figure 9a), (4,16)-POMECY with DMPC (Figure 9b), and pure (4,16)-POMECY (Figure 9c) prior to and after polymerization. The nonpolymerized mixtures exhibit slightly broadened transitions, and the liquidus and solidus lines

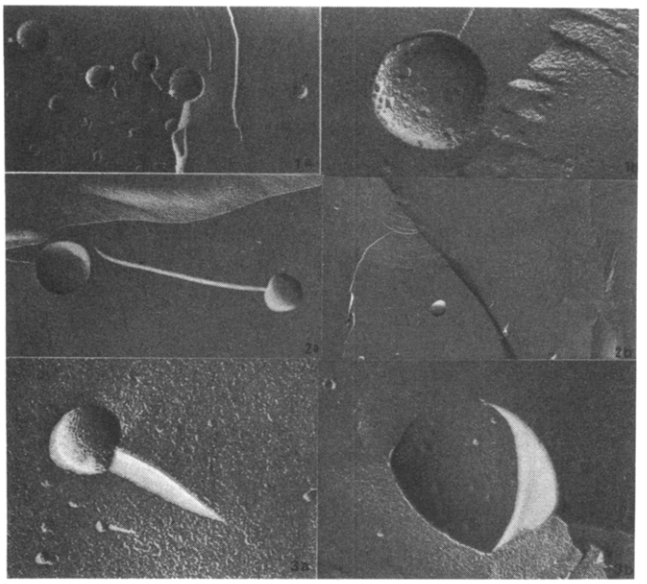


Figure 10. Freeze fracture electron micrographs of (4,14)-POMECY/DMPC mixed vesicles. (a) (left column) Before polymerization. (b) (right column) After photopolymerization for 5 min. Row 1: 25:75 mixture of (4,14)-POMECY and DMPC. Row 2: equimolar mixture of (4,14)-POMECY and DMPC. Row 3: 75:75 mixture of (4,14)-POMECY and DMPC. Note that small domains are formed within the bilayer after polymerization while the vesicle surfaces are smooth in the nonpolymerized state. In the case of the equimolar mixture, the large vesicles decay into small ones. All samples were (rapidly) frozen from 25 °C. The width of each image corresponds to 7 μm.

can be seen only if the chain lengths differ (cf. Figure 9a). Polymerization leads to a braod band centered at about 40 °C for both DMPC and DPPC as monomeric component. This band can therefore be attributed to the polymerized (4,16)-POMECY. In addition, a broad band remains at about the transition temperature of the pure lipid, that is, at about 40 °C for Figure 9a and at about 25 °C for Figure 9b. It is attributed to the phospholipid fraction (DPPC in the case of Figure 9a and DMPC in Figure 9b) containing some POMECY.

The appearance of two well separated bands after polymerization suggests that the vesicles become unstable and decompose into a fraction of vesicles of the pure monomer (DMPC or DPPC) containing some nonpolymerized POMECY (or small oligomers) and a fraction composed mainly of large polymerized amphiphile (exhibiting a transition at about 40 °C). Evidence for such a heterogeneous phase separation is indeed provided by freeze fracture electron microscopy and phase contrast microscopy as shown in Figures 10 and 11. Figure 10 exhibits freeze fracture microcraphs of 25:75, 50:50, and 75:25 mixtures of (4,14)-POMECY and DMPC. The vesicles exhibit clearly a smooth surface before polymerization. After polymerization, the formation of small domains of approximately 50 nm in diameter (cf. right part of Figure 10) is observed. A closer comparison of the shadow of the vesicle and the domains shows that the latter are always bulged toward the inside of the mother vesicle. Moreover, a closer inspection of the vesicle size distribution shows that after polymerization a much larger number of very small vesicles is found (not shown) which are not observed before polymerization. These exhibit an average diameter of ≈40 nm. This value corresponds rather well with the average diameter of the domains. This leads to the conclusion that most of the polymerized lipid was detached from the mother liposome in the form

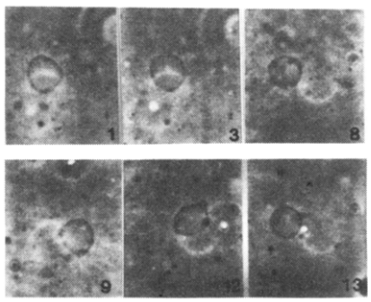


Figure 11. Phase contrast micrograph of giant vesicle of 70:30 mixture of (4,14)-POMECY and DMPC observed during polymerization. The polymerization proceeds from left to right. The small buds formed during photopolymerization are pointing to both the outside and the inside of the mother vesicles. Note that the giant vesicle becomes smaller and that many small vesicles appear in the buffer. Vesicle diameter is about 20 μm.

of small vesicles. As argued below, these vesicles could well be formed by a single large macrolipid containing some monomeric lipid.

The destabilization of the giant vesicles after polymerization is further demonstrated in Figure 11, which shows a series of phase contrast micrographs of a giant mixed vesicle of (4,14)-POMECY and DMPC taken during photochemically induced polymerization. Small buds are continuously formed pointing both toward the inside and outside of the vesicles. A closer inspection of the micrograph in Figure 11 shows further that the mother vesicle simultaneously becomes smaller, which is attributed to the detachment of small vesicles.

Discussion

Photochemically Induced versus Initiator-Induced Polymerization. The present work has shown that large macromolecular amphiphiles with monomer numbers of $N \approx 10^4$ can be produced in two-dimensional solutions both by photochemical- and initiator-induced polymerization. Clearly, the photochemical technique is not well suited to polymerize amphiphiles in vesicle suspensions since the newly formed polymeric amphiphiles are rapidly cleaved by photochemical decomposition. In order to avoid extensive photodecomposition, the irradiation time should be as short as possible. This requires (i) that the vesicles in suspension be small and unilamellar and (ii) that the total lipid concentration is low in order to minimize the scattering-induced attenuation of the light passing through the reaction cell. Continuous stirring might help to improve the situation. The strong reduction of the yield of large polymers with increasing sample turbidity in the present study provides an explanation for previous findings that photopolymerization of concentrated vesicle suspensions yielded only small oligomers[4,5] while high molecular weight macrolipids of POMECY with $N \approx 300$ could be formed by irradiation of single giant vesicles under a microscope.[1,7]

High molecular weight macrolipids can be formed most effectively by initiator-mediated polymerization. The polymer length distribution is shifted toward shorter chains with increasing initiator-to-POMECY mole ratio. This is of course a consequence of the increase of the number of reactions started simultaneously. As suggested by Figure 5c, a rather sharp distribution of long chains is obtained if the initiator

concentration is just sufficient to initiate polymerization within a reasonable time (about 1 h).

While this paper was in preparation, two excellent quantitative studies of radical-induced polymerization of an amphiphile where the reactive group is associated with the hydrocarbon tails of the lipid appeared.[11,12] The average degrees of polymerization varied between 50 and 2000 monomers. It was shown that the size decreased with the initiator concentration, in agreement with our more qualitative results. It is thus well established that large polymers may be produced in two-dimensional lipid solutions.

Threshold POMECY Concentration and the Radical Lifetime. A very interesting result is the formation of a rather large relative amount of large polymers at POMECY concentrations as low as 25%. This holds for both the photochemical- and initiator-mediated reactions. This shows that the diffusion-controlled polymerization reaction in two-dimensional solutions is very effective. This is further verified by the finding that the size distribution of POMECY polymerized in three-dimensional (dioxane) solution is considerably shifted to shorter chains as compared to the distribution obtained by polymerization in vesicles.

The length of polymer is limited by (1) the lifetime, t_r, of the radical at the end of the chain growing according to

$$\text{I}-\text{M}^* + \text{M}_n \leftrightarrow \text{I}-\text{M}^*_{n+1}$$

(where M_n is in general a monomer but could also be an oligomer) and (2) reactions leading to the termination of chain elongation such as (i) combination of two growing chains, (ii) combination of a growing chain end with an initiator radical, and (iii) disproportionation of polymers. As shown by Sells and O'Brien,[11] the second mechanism prevails at high initiator concentration.

From the value of the threshold concentration of POMECY above from which large polymers are formed, one can estimate the lifetime of the radical at the chain end as follows: The average collision time between two reaction partners (e.g., a POMECY radical and a POMECY molecule) is about

$$t_c = \frac{d_{pp}^2}{4D_{lat}}$$

where d_{pp} is the average distance between the POMECY molecules and D_{lat} is the lateral diffusion coefficient. The threshold concentration of (4,16)-POMECY at which polymerization proceeds is 5–10 mol %, corresponding to the mole fraction $x_p = 0.05–0.10$. The average distance between the reactants is therefore

$$d_{pp} = \frac{\sqrt{A_L}}{\sqrt{x_p}}$$

where A_L is the area per lipid molecule. It thus follows that just above the threshold concentrations the collision time is

$$t_c = \frac{A_L}{4x_p D_{lat}} \approx 1–3 \times 10^{-7}\ \text{s}$$

where the value of $D_{lat} = 10^{-7}$ cm^2/s[8] was assumed. This is an upper limit of the lifetime, t_R, of the POMECY radicals.

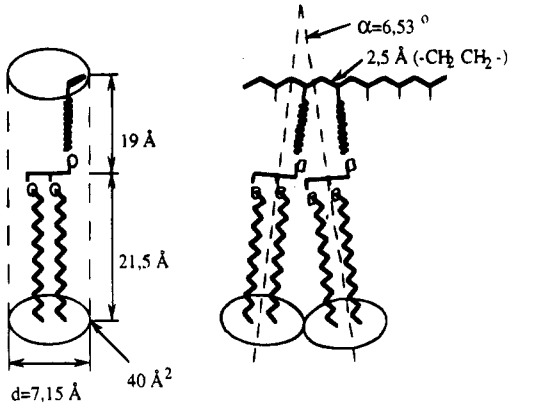


Figure 12. Topological model of monomer of polymerizable amphiphile and of two interconnected molecules.

A remarkable finding is that in nearly all cases well-separated low ($N \sim 100$) and high ($N \sim 10^4$) molecular weight fractions are found (cf. Figure 2b), in particular in the case of photochemical polymerization. A likely explanation is as follows. Due to the low concentration of photochemically produced radicals and the small size of the vesicles, only one or two chains are expected to start to grow simultaneously in each vesicle. These initially formed chains grow to maximum length before new chains are initiated. The effective monomer concentration is considerably reduced, and the lengths of the newly formed polymers are therefore expected to be much smaller. Thus, according to Figure 7 only small polymers are formed at <20 mol % of POMECY. Chain termination may occur by any of the above chemical mechanisms, although our data do not allow to distinguish between these. Another possibility, suggested by the electron and phase contrast microscopic studies (cf. Figures 10 and 11), is that the domains formed by the large polymerized amphiphiles bud and detach from the mother vesicle before polymerization is completed.

Phase Separation and Vesicle Stability. One aim of embedding POMECY in DMPC bilayers was to stabilize large vesicles and to prepare two-dimensional polymer solutions.[1] The present experiments show conclusively that the formation of large polymers is associated with lateral phase separation followed by domain formation. Unfortunately, the domains are not stable, and local budding and fission of the buds occur. This budding is a consequence of spontaneous curvature of the domains enriched in macrolipid.[12] As shown in Figure 12, the average distance between the ends of the hydrophilic head groups of two interconnected POMECY molecules is only 2.5 Å and is thus considerably smaller than the average cross-section occupied by the associated hydrocarbon chain pair (about 7 Å). This constraint is expected to lead to a negative spontaneous curvature after polymerization, as indicated in Figure 13. It is further expected that in the presence of monomeric lipid (e.g., DMPC) the local elastic strain within a domain can be relaxed by accumulation of this component in the opposite monolayer. Evidence for this view is provided by the freeze fracture experiments which show that the small domains of diameter <100 nm are generally bent toward the inside of the vesicles. Since monomeric POMECY has a much larger head group than DMPC, it is expected to enrich in the outer monolayer of small vesicles during vesicle preparation. Since the outer monolayer contracts within the head group region during polymerization, polymerized domains will bulge toward the inside of the mother vesicle. In the case of giant vesicles, the phase contrast micro-

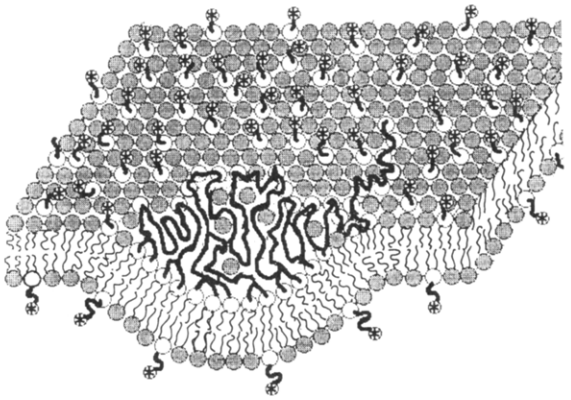


Figure 13. Schematic view of budding of polymerized domains toward the inside of a giant vesicle owing to contraction of the head group region of the polymerized domain.

scope observations of Figure 11 show that the small buds point toward alternatingly the outside and inside of the other vesicle. This is expected since in giant vesicles the two components are more randomly distributed between the two monolayers and budding can occur in both directions.

The domains observed in the freeze fracture electron micrographs have diameters of 50−100 nm. Provided they were composed of pure macrolipid, this would correspond to about 3000−14 000 monomers. Interestingly, these numbers correspond well with the degrees of polymerization of the large polymers, suggesting that each domain is composed in general of a single macrolipid containing some DMPC.

Concluding Remarks

The present study has shown that polymerization in two-dimensional solutions is very effective. Large macromolecular amphiphiles can be prepared by both photochemically initiated and initiator-initiated polymerization. The former technique is not well suited for vesicle suspensions since the newly formed polymers are rapidly destroyed photochemically. For that reason, smaller polymers with $N \sim 300$ were found in our previous study. It is, however, well suited to polymerize amphiphiles in single giant vesicles by irradiation under a microscope since the initial stages of polymer formation can be observed (Figure 11) and the time of exposure of the vesicle to irradiation can thus be minimized. The photolytic decomposition could most probably be further reduced by irradiation with narrower bandwidth or by photochemical cleavage of initiators absorbing at longer wavelengths. The initiator-mediated polymerization yields high molecular weight polymers and offers several advantages. The most important is that the polymer size can be controlled by variation of the initiator concentration. The present work shows that another intriguing way to control the polymer size is to adjust the composition of the mixtures of polymerizable and nonpolymerizable lipids.

Polymerization in two dimensions produces longer polymer chains than in three-dimensional solutions as shown by comparison of POMECY polymerization in vesicles and in dioxane. One possible reason is that the strong coiling of growing polymers in bulk solutions impedes the access of new monomers to the growing end of the chains. As shown by computer simulation experiments,[7,13] macromolecules embedded in two-dimensional solutions are more stretched and the accessibility of the growing end by new monomers is less severely impeded. This fact could be exploited in order to control the polymer sizes by lateral phase separation within the vesicle prior to polymerization. As is well known, domains of narrow size distribution can be achieved by control of the phase separation process.[13] Provided all polymerizable molecules in a domain can be polymerized, the degree of polymerization can be controlled.

One main aim of our studies of partially polymerized vesicles was to prepare mechanical models of cell plasma membranes.[13] Due to the inherent instability of giant vesicles after polymerization, our efforts have to date failed. One possible way to overcome this problem could be to first produce short chains by interconnecting amphiphiles via the head groups (using water soluble initiator) and then cross-link these oligomers in a second step. This could be achieved if some of the amphiphiles carried polymerizable groups at the hydrocarbon chains[1] which could be cross-linked in a second step by adding initiator which is soluble only in hydrophobic solvents (that is, within the bilayer).

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List of Abbreviations

POMECY	1,2-*O*-diacylglycero-3-[2-[2′-(methacryloyloxy)-tris(ethyleneoxy)]ethyl]phosphoric acid diester sodium
DMPC	dimyristoylphosphatidylcholine
ACVA	4,4′-azobis[4-cyanoavaleric acid]
GPC	gel permeation chromatography
HPLC	high-pressure liquid chromatography
QELS	quasi-elastic light scattering
PMMA	poly(methyl methacrylate)
N	degree of polymerization
m	mole ratio of initiator to POMECY

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