New Lipopolymers for the Fixation of Lipid Bilayers

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SUMMARY: Here we report the synthesis of end functionalized lipopolymers. For this purpose different lipoinitiators with a bromo group have been prepared, based on monoalkyl and dialkyl N-substituted 2-bromo-propionamides. Theses lipoinitiators could then be used as initiators for atom transfer radical polymerization of acrylamides. Different lipopolymers were synthezised. The polymer back bone was either a homopolymer or a copolymer. Thin polymer films of 20 Å thickness of the copolymers were self-assembled onto surfaces and, finally, vesicle fusion on these polymer cushions was studied.

Introduction

Surface modification is a major research object of great interest. Especially the preparation of thin polymer films on surfaces is an important field. Major developments have been performed and first applications come on the market, e.g. superhydrophobic surfaces. Combining the possibility of tunable surface properties and biological systems, such as lipid membranes, opens a new research area. One project is the realization of new bio-sensors, which might lead to possible applications in the growing field of bio-technology. In order to achieve such a device, lipid bilayers need to be fixed onto a surface. As Sackmann et.al [1], Ringsdorf et.al [2] and others [3,4] have shown, such systems can be build up by vesicle fusion onto different supports.

So far several successful attempts to prepare lipid membranes on surfaces have been reported. Both the fusion of vesicles onto hydrophobic self-assembled monolayers (alkylthiols) [5] and the fusion onto hydrophilic spacers (either oligomer or polymer) [4,6] are promising attempts. However, it is still a challenge to develop new systems in order to improve the stabilization of lipid bilayers on polymer supports. We have focused on the synthesis of lipopolymers [6,7,8] and here we present new lipopolymers prepared from lipophilic initiators.

The results are based on the idea to synthesize a defined structure of lipophilic polymers. The general idea is to have a hydrophilic polymer with two different end groups. One hydrophobic

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end group, which acts as a lipophilic anchor for the lipid bilayer, and one end group, which acts as a surface anchor, as shown schematically in Fig. 1.

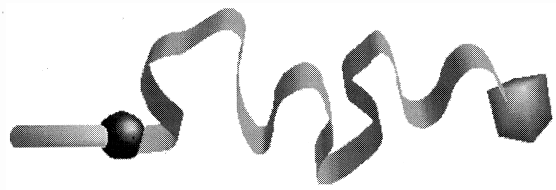


Fig. 1: Schematic picture of the desired lipophilic polymer. On the left side is the lipophilic anchor and on the right side (cubus) the surface anchor, which are joined by a hydrophilic polymer backbone.

On the way to reach this goal, we have synthesized one end functionalised lipopolymers, which we present here. First, different lipophilic initiators from 2-bromo-propionic acid were synthesized. These were then characterized and their lipophilic behavior was studied. Second, the polymers were synthesized by atom transfer radical polymerisation [9,10,11] using the lipophilic initiators . In order to prepare a polymer support, thin polymer films have been self-assembled onto negatively charged surfaces. And, finally, vesicle fusion on these supports was briefly investigated.

Results and Discussions

Initiator synthesis

First, we have synthesized a model initiator N-isopropyl-2-bromo-propionamide **7** in order to study the polymerization conditions. The product could be obtained from isopropylamine and 2-bromo-propionacid bromide in THF. After recrystallisation from hexane, white needles were obtained in good yields.

All lipophilic initiators were synthesized in the same way, as shown in Fig. 2. First, the alkylacid was coupled with the alkylamine to an di-alkylamide. The amidation was performed using carbonyldiimidazol as an auxiliar. All di-alkyamides gave good yields in the range of 80 %.

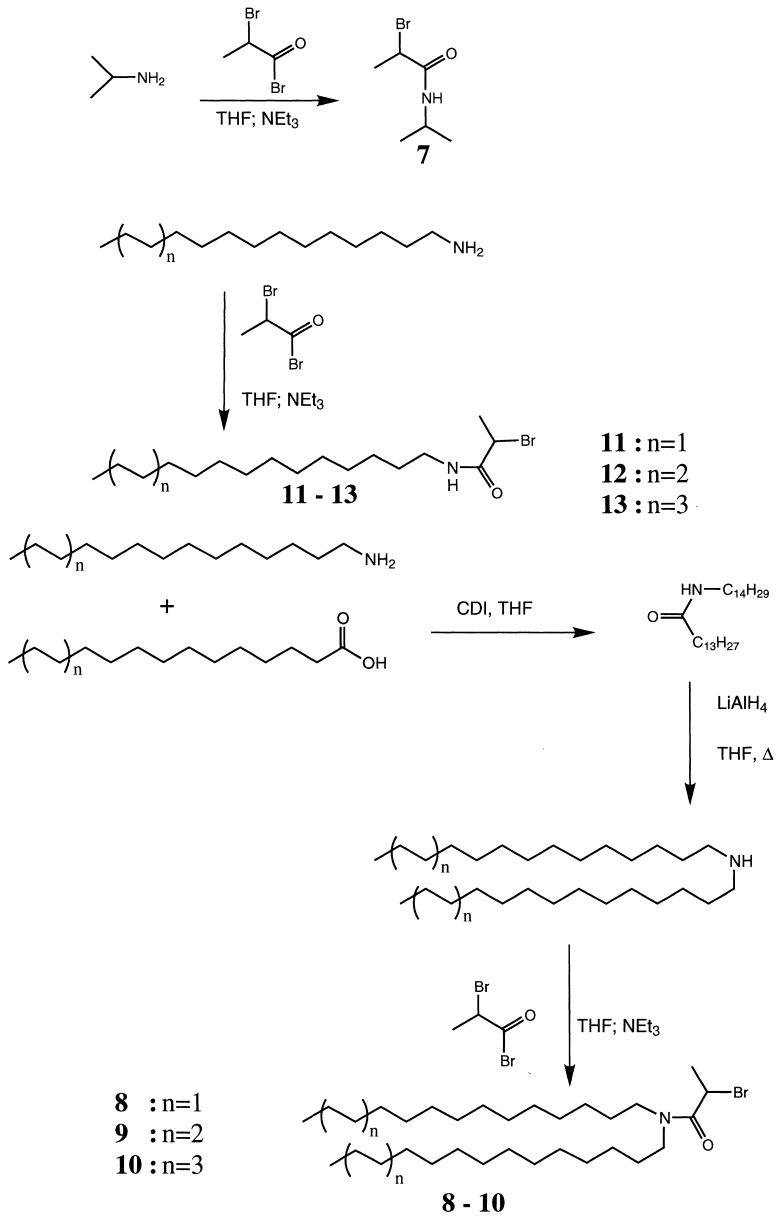


Fig. 2: Synthesis of lipoinitiators.

In a second reaction these amides were reduced to the secondary amines. In all reductions the reducing agent was $LiAlH_4$ in THF. The mixture was allowed to react for at least 12 hours.

Purification by chromatography yielded white powders. TLC, NMR and IR prooved the reduction to the amine.

The final initiator was synthesized by amidation of 2-bromopropionacid bromide with the secondary alkylamines prepared above, or primary alkylamines in chloroform. When possible, all amidations were cooled during the reactions. Depending on the amine, purification was either performed by chromatography or recrystallisation. The initiators synthesized are summarized in table 1.

Table 1: Summary of (lipo)initiators.

productnumber	name	yield [%]
7	N-isopropyl-2-bromo-propionamide	57
8	N,N-ditetradecyl-2-bromo-propionamide	45
9	N,N-dihexadecyl-2-bromo-propionamide	48
10	N,N-dioctadecly-2-bromo-propionamide	42
11	N-tetradecyl-2-bromo-propionamide	53
12	N-hexadecyl-2-bromo-propionamide	55
13	N-octadecly-2-bromo-propionamide	50

Initiator Characterization

To get a first feeling of the amphiphilic behavior of the one end functionalized polymers, we have measured isotherms of all lipoinitiators at the air water interface. All isotherms have been recorded at 20°C. They are plotted in Fig. 3.

The lipoinitiators **8-10** and **11-13** differ only in the chain length of the alkyl chains. As expected, this variation from 14 to 18 carbon atoms has no effect on the isotherms. Therefore, only one isotherm per initiator row (**8-10** and **11-13**, respectively) is plotted in Fig. 3a and 3b.

As can be seen directly, there is a huge difference between the N,N-dialkyl initiators **8-10** and N-monoalkyl initiators **11-13**. Initiators **11-13** show a collapse point at an area of 41 $Å^2$ (Fig. 3b). This area is in agreement with the size of the head group, as estimated from a molecular modelling program (40 $Å^2$) [12]. It is far too large for a dense packing of the alkyl chains (20 $Å^2$).

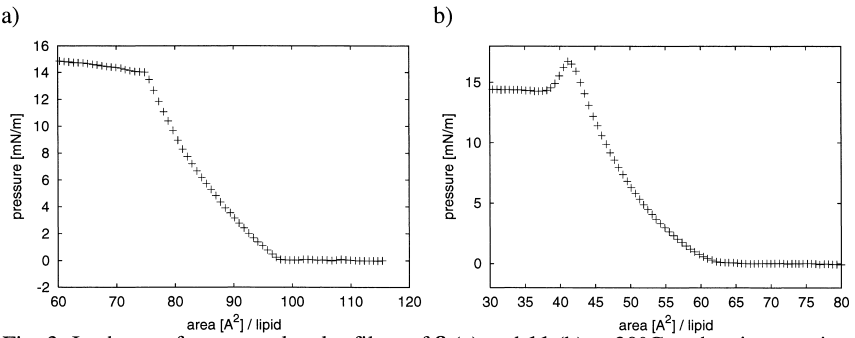


Fig. 3: Isotherm of monomolecular films of **8** (a) and **11** (b) at 20°C at the air water interface. The isotherms of **9** and **10** are nearly identical to **8** (a); the isotherms of **12** and **13** are nearly identical to **11** (b).

However, the initiators **8-10** show a collapse point at an area of 75 Å (Fig. 3a). Their head group requires only about 45 $Å^2$ at the air water interface, as estimated from a molecular modelling program [12]. A close packing of two alkyl chains would lead to about 40 $Å^2$. Both values are smaller than the measured collapse point and it seems that these initiators are not able to form stable densed packed monolayers.

In addition it has to be mentioned that all monolayers collapse at a relative low pressure of about 16 mN/m. This is probably due to the fact that the lipoinitiators miss a really polar head group and are not real amphiphiles.

Polymer synthesis

All polymerizations were based on the ATRP mechanism [9,10,11]. We are aware of the problem that ATRP of acrylamides is not a perfectly controlled polymerisation [13]. However, we are aiming at low molecular weight polymers. All polymerizations presented are either performed in water or in a THF/water mixture.

A model polymerization was performed with the model initiator **7** in water with acrlyamide as a monomer. The reaction was conducted in a sealed NMR tube. NMR spectra were taken every minute and are the mean value of 4 scans taken within 20 seconds. The spectra were standardized against the water peak, not shown here. The polymerization occured very fast. Within 5-10 minutes the reaction was finished, depending on the reaction temperature. The plot of the logarithm of $[M]_0/[M]_t$ versus time, with $[M]_0$ as the monomer concentration at time 0 and $[M]_t$ as the monomer concentration at time t, (Fig. 4) shows a linear behavior describing a controlled polymerization behavior [10].

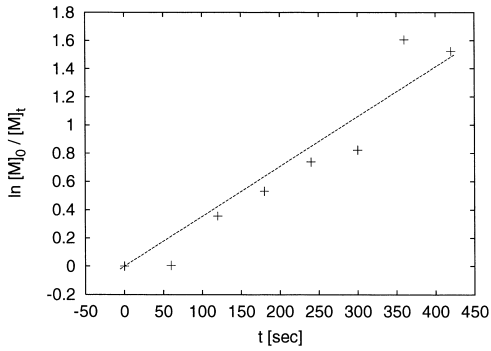
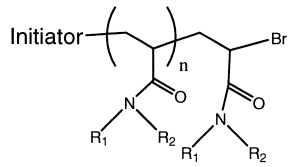


Fig. 4: Kinetic analysis of acrylamide polymerization.

The polymers were finally precipitated in acetone, centrifugated, and dried in vaccuum. It is a major problem to remove the copper salt from the polymer solution. After many attempts to apply the published procedures to remove copper [11], we found that dialysis seems to be the best working method. Since our polymers are all water soluble, the dialysis is easy to perform. Polymers were dissolved in water and were three times dialyzed against 3 % NH_3-solution and then three times against ultrapure water. Again, by precipitation in acetone the pure polymers could be obtained.

Table 2: Selection of typical polymers.



P1 - P4

polymer	initiator	monomers	P_n
P1a	**7**	acrylamide	50
P1b	**7**	acrylamide	100
P2	**9**	acrylamide	50
P3	**11**	acrylamide	50
P4	**11**	acrylamide/N-(3-dimethylaminopropyl)-acrylamide	50

Using the parameters determined by the model polymerization, we have prepared one end functionalized polymers. The polymerization was started from the lipoinitiators. It was possible to homopolymerize different acrylamide monomers, e.g. acrylamide, N-isopropyl-acrylamide. The degree of polymerization (P_n) was calculated by end group analysis using NMR. Polymers with P_n between 50 to 100 have been synthesized depending on the ratio of initiator to monomer calculated for a living polymerization.

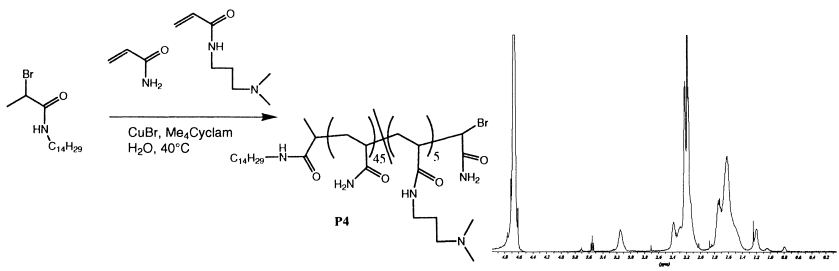


Fig. 5: Copolymerization and the corresponding NMR spectra.

Also we could statistically copolymerize different acrylamide monomers, e.g. acrylamide and N-(3-dimethylaminopropyl)-acrylamide. The copolymers have been analyzed by NMR and we could confirm the ratio of monomers incorporated in the polymer (Fig. 5).

Polymeradsorption and vesicle fusion

The copolymer **P4** described in the previous part could be protonated in acidic media since it has tertiary amine groups. In the protonated state the polymer can be self-assembled as a polyelectrolyte on a negatively charged surface following the concept of Decher et.al [14]. The negative surface was a gold surface modified with 3-mercaptopropan sulfonic acid.

The self-assembly process was monitored by surface plasmon resonance (SPR) [6]. A typical kinetic curve of the self-assembly process is shown in Fig. 6a. Within the first minutes the film thickness increased rapidly. Thereafter only a small continuous thickness increase could be observed. Rinsing with water gave a stable signal and a final film thickness of 20 Å could be calculated.

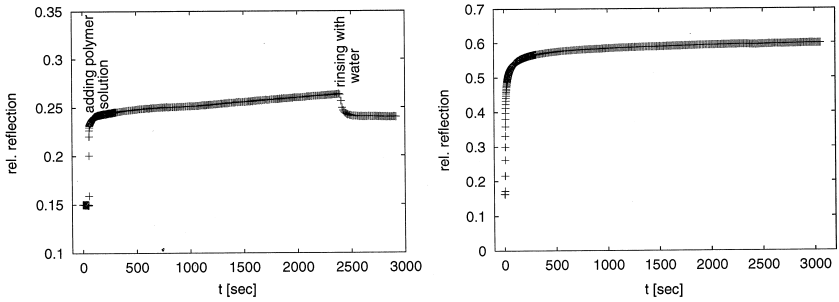


Fig. 6: a) Self-assembly of lipopolymer and b) vesicle adsorption on this polymer support.

The self-assembled thin lipopolymer film is capable of interacting with vesicles. We have used vesicles prepared by extrusion using DMPC. Their preparation is already described elsewhere [6]. DMPC vesicles with a diameter of about 50 Å have been brought into contact with the polymer surface. A fast increase in thickness could be observed by SPR (see Fig. 6b). Within 5 minutes most of the thickness increase has happened. After that only a small thickness increase could be observed. The final thickness increase was 62 Å. This is larger than the increase in thickness expected from the adsorption of a lipid bilayer (50 Å). In agreement with other results we asume that the vesicles are only adsorbed and not yet fused into a lipid bilayer [15].

Experimental Part

General. Chemicals were purchased from Sigma-Aldrich and were used without further purification unless otherwise noted. The ultrapure water used for all cleaning steps and as solvent for the adsorption was purified by reverse osmosis (Milli-Q, Millipore GmbH).

Acrylamide was recrystallized from $CHCl_3$. N-(3-Dimethylaminopropyl)-acrylamide was distilled under reduced pressure. CuBr was stirred over acetic acid , filtered off, washed with methanol, and dried in vacuum.

^{1}H-NMR spectra were recorded using $CDCl_3$ on a 400 MHz Bruker spectrometer. Chemical shifts (δ) are given in ppm relative to TMS. IR spectra were recorded on a Nicolet Protegé. Wave numbers (ν) are given in cm^{-1}.

Isotherms were measured on a NIMA film balance with ultrapure water as the subphase. SPR curves were recorded in a standard Kretschmann configuration which has been described elsewhere [7].

Polymer self-assembly was performed from a water solution. Vesicles were prepared by extrusion through a 50 nm polycarbonate filter as described earlier [6].

Preparation of Amides 1-3. 0.02 mol of the respective acid was dissolved in 10 mL dry chloroform. Under nitrogen atmosphere 0.02 mol of carbonyldiimidazol was added. After the CO_2 formation was finished the solution was stirred for half an hour. Then a solution of 0.02 mol of the respective amine dissolved in 10 mL dry chloroform was added. The reaction was stirred for another three hours. The mixture was extracted with water and the organic phase was dried over Na_2CO_3. Evaporation of the solvent resulted in a white residue. Recrystallisation in ethanol gave the desired product (80 % yield). ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 6H, CH_3); 1.27 (44 [48 or 52] H, CH_2); 1.46 (2H, CH_2); 1.57 (2H, CH_2); 2.10 (2H, $COCH_2$); 3.19 (2H, $NHCH_2$); 5.41 (1H, NH). IR ν : 3309 (N-H); 2954, 2914, 2849 (C-H); 1635 (Amide I); 1544 (Amide II); 1471 (C-H); 715 (C-H rocking).

Preparation of Amines 4-6. 0.01 mol of the respective amide was suspended in 250 mL dry THF. $LiAlH_4$ was added in small portions to the mixture. After all $LiAlH_4$ was added, the mixture was allowed to reflux for 12 hours. Then the mixture was cooled down and quenched with small portions of water. Resulting precipitated $Al(OH)_3$ was filtered off and extracted three times with warm THF. The combined solutions were concentrated, diluted with $CHCl_3$, and extracted with water. Evaporation of the solvent resulted in white residue. Purification of the product was performed by flash chromatography on Silicagel using $CHCl_3$ as the mobile phase (50 % yield). ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 6H, CH_3); 1.24 (44 [48 or 52] H, CH_2); 1.50 (4H, CH_2); 2.60 (4H, $NHCH_2$). ^{13}C NMR ($CDCl_3$) δ: 14.1 (CH_3); 22.7 (CH_3-CH_2); 27.4, 29.7, 31.9 (CH_2); 49.9 ($NHCH_2$). IR ν : 2917, 2849 (C-H); 1472, 1461 (C-H); 1129; 719 (C-H rocking).

Preparation of N-isopropyl-2-bromo-propionamid 7. 38 mmol N-isopropylamine was dissolved in 10 mL dry chloroform. 19 mmol 2-bromo-propion-acid bromide was added as a solution in dry chloroform. After reaction time of 3 hours, the mixture was washed with water and the solvent evaporated. Purification was performed by recrystallisation from heptane (yield 57 %). ^{1}H NMR ($CDCl_3$) δ: 1.16 (d, 6H, $CH(CH_3)_2$); 1.84 (d, 3H, CH_3CHBr); 4.02 (m, 1H, $CH(CH_3)_2$); 4.34 (m, 1 H, CH_3CHBr); 6.15 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 22.3, 23.2 (CH_3); 42.2, 45.6 (CH); 168.4 (C=O). IR ν : 3269, 3085 (N-H); 2972, 2928 (C-H); 1645 (Amide I); 1553 (Amide II); 1368; 1219; 1193; 1160; 1070; 988; 910; 773.

Preparation of 2-Bromo-propionamides 8-13. 1 mmol of the respective amine **4-6** or the primary amines was dissolved in 5 mL dry $CHCl_3$. Triethylamine and 2-bromopropionicacid

bromide were added slowly one after another as a solution in $CHCl_3$. Afterwards the solution was diluted with $CHCl_3$ and extracted with water. The organic phase was then dried over Na_2CO_3 and the solvent evaporated. Purification was done by flash chromatography with $CHCl_3$ or recrystallisation (50 % yield). ^1H NMR ($CDCl_3$) δ: 0.87 (t, 6H, CH_3); 1.25 (44H, CH_2); 1.53 (4H, CH_2); 1.79 (d, 3H, CH_3CHBr); 3.10 (m, 2H, CH_2NH); 3.41 (m, 2H, CH_2NH); 4.50 (m, 1H, CHBr). ^{13}C NMR ($CDCl_3$) δ: 14.1 (CH_3); 21.8, 22.7, 26.9, 27.2, 29.4, 31.9, 38.6, 46.6, 48.2 (CH_2); 168.8 (C=O). IR ν : 2918, 2848 (C-H); 1652 (C=O); 1460, 1426 (C-H); 1136; 724 (C-H rocking).

General Polymerization Procedure. Polymerization was performed in a 50°C warm THF/water mixture using 1,4,8,11-tetra-aza-1,4,8,11-tetra-methyl-cyclo-tetra-decane as a ligand for CuBr. Initially the initiators **7-13** were added to a solution THF/water (9:1). After degasing the monomers dissolved in water were added continuously. Polymers were precipitated after 10 minutes in acetone. Polymers were then purificated by dialysis (yield 80 %). Molecular weights were determined by end group analysis from the NMR spectra.

Conclusion and outlook

Lipoinitiators can be prepared on the basis of 2-bromo-propionamides. They are able to initialize the atom transfer radical polymerization of acrylamides. We could show that ATRP is a powerful technique to synthesize lipopolymers, especially when a controlled polymer structure is the goal. So far we reached the goal to polymerize one end functionalized lipopolymers. The next step will be the functionalization of the other end group with a surface anchor group.

Nevertheless, the one end functionalized polymers can be self-assembled onto negatively charged surfaces and vesicles can be adsorbed onto these thin polymer films.

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