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Amino-functionalized single-chain bolalipids: Synthesis and aggregation behavior of new basic building blocks☆

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ABSTRACT

Herein, we report the synthesis of two novel, amino-functionalized single-chain bolalipids and, based on those, a general synthetic approach for the insertion of various carboxylic acids into the bolalipid headgroups, e.g. α-lipoic acid for one-dimensional fixation of gold nanoparticles, sorbic acid for polymerization experiments, or lysine for the use in gene delivery systems. The temperature- and pH-dependent self-assembly of amino-functionalized bolalipids into nanofibers and micelles was investigated by differential scanning calorimetry (DSC), transmission electron microscopy (TEM) and dynamic light scattering (DLS). Rheological measurements were used to describe the macroscopic behavior of the formed temperature switchable hydrogels that can be fine-tuned for drug delivery applications. We showed that the viscoelastic properties of the hydrogel strongly depend on ionic interactions between bolalipid headgroups as well as on the ability to form hydrogen bonds.

1. Introduction

Over the last 30 years, representatives in the class of bipolar lipids (bolalipids) emerged as basic structures for wide-spread modifications enabling versatile applications in the field of bioscience. As substantially reviewed, those bolalipids, originating in the membrane lipids of certain species of *Archaea*, connect two hydrophilic headgroups via a lipophilic spacer mainly consisting of one or two alkyl chains [1]. The membrane-spanning nature and, hence, membrane-stabilizing properties of these bolalipids are responsible for the outstanding stability of the *Archaea,* and those qualities make them attractive candidates for the use in vesicular drug delivery systems [2] or for the stabilization of supported biosensor devices [3]. With regard to their challenging synthesis, numerous attempts were made to simplify their chemical structure resulting in the preparation of single-chain bolalipids [4]. Unexpectedly, this class of simplified bipolar lipids forms novel aggregate structures, e.g. nanofibers and nanoparticles of self-assembled bolalipids, which are uncommon for the natural compounds [5,6].

Recently, we reported the synthesis and aggregation behavior of symmetrical, single-chain polymethylene-1,ω-bis(phosphocholines) with alkyl chain lengths of 22 to 32 carbon atoms and two phosphocholine headgroups bilaterally attached [7]. These simplified *archaeal* model lipids self-assemble in water at room temperature into well defined, long and flexible nanofibers, forming a dense network, which gels water very efficiently — even at very low concentrations of 0.3 mg/ml [8,9]. Within the fiber, bolalipid molecules are arranged side by side, but twisted relative to each other due to the bulky headgroups. These packing constraints lead to a helical superstructure of the nanofibers [10]. Since the phosphocholine headgroups are not able to form intermolecular hydrogen bonds, the self-assembly process is exclusively driven by *van-der-Waals* interactions between the long alkyl chains and, thus, it is strongly related to temperature: Above a certain transition temperature, which depends on the lengths of the alkyl spacer, the nanofibers reversibly transform into smaller aggregates like spherical micelles or discs and the gel character of the suspensions is completely lost [7].

An additional stabilization can be achieved by replacing the phosphocholine with phosphodimethylethanolamine headgroups, which introduces further hydrogen bonds [11,12]. The presence of dissociable H-atoms induces the formation of pH-sensitive bolaform hydrogelators, which might be of importance for drug delivery systems. Protonable moieties can also be realized by amino-

☆ Dedicated to Prof. Alfred Blume on the occasion of his 65th birthday.

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functionalized headgroups. However, the first attempt to synthesize an amino-functionalized single-chain bipolar lipid using phosphorylation of dotriacontane-1,32-diol and a subsequent quarternisation with *N*,*N*-dimethylethylenediamine failed due to the limited purification feasibility of the final reaction mixture [13].

In the work presented here, we seized on this last concept: We developed the synthesis of two novel amino group containing bolalipids using both a one-side protected *N*,*N*-dimethylethylenediamine for the quarternisation reaction and the *click*-chemistry in combination with a 2-azidoethylamine acting as linker. These amino-functionalized bolalipids represent new basic building blocks for tailor-made bolalipids carrying various functional groups. Both building blocks and a selection of modified bolalipids as well as their potential applications are illustrated in Fig. 1.

The presence of amino-functionalized headgroups allows the implementation of 1) α-lipoic acid for the one-dimensional fixation of gold nanoparticles on bolaform nanofibers [10] with a possible quantum dot behavior [14] and 2) sorbic acid for the polymerization of the headgroup region in order to stabilize the structure of the fibers. Moreover, 3) basic amino acids like lysine can be used enabling the fixation of DNA and/or the stabilization of the DNA-transporting liposomes which might be employed in gene delivery systems [15].

Further headgroup modifications are under investigation. They are mentioned here to show the diversity of feasible functionalization reactions. For instance, 1) the insertion of a fluorescence label [16] could yield information about the dynamics of the bolalipids within the self-assembled nanofibers. The coupling of 2) low-molecular weight peptides might be of interest for applications addressing biological sensing [17], and 3) the binding of polyethoxy moieties should result in nanofibers with hydophilized surfaces.

In this work we present the synthesis of the amino-functionalized bolalipids AEPC-C32-AEPC and AETPC-C32-AETPC. Based on those, we propose a general synthetic approach for the insertion of various carboxylic acids in the headgroup of these bolalipids. The temperature- and pH-dependent self-assembly of AEPC-C32-AEPC and AETPC-C32-AETPC in water was investigated in order to test the stability range of the formed aggregates and to adjust the viscoelastic properties of the formed hydrogels. Therefore, we studied pure and mixed bolalipid suspensions with the unmodified dotriacontane-1,32-diyl-bis[2-(trimethylammonio)ethylphosphate] (PC-C32-PC) by means of differential scanning calorimetry (DSC), transmission electron microscopy (TEM) and dynamic light scattering (DLS). The macroscopic behavior of the formed hydrogels was described by rheological measurements.

2. Materials and methods

2.1. Materials and synthesis

Sodium chloride, sodium carbonate and sodium hydrogen carbonate (p.a. grade) were purchased from Carl Roth GmbH&Co (Karlsruhe, Germany). Substances for syntheses were purchased from Sigma Aldrich Co. and were used without further purification. β-Bromoethylphosphoric acid dichloride was prepared according to the literature [18], and dotriacontane-1,32-diol as well as PC-C32-PC were synthesized according to the procedures described previously [7]. All organic solvents used were purified and dried. The purity of all compounds was checked by thin-layer chromatography (TLC; obtained from Merck). The purification of the final bolalipids was carried out by middle pressure liquid chromatography (MPLC; Büchi) or conventional column chromatography using silica gel (Merck, 0.032–0.060 mm). Detailed analytical data concerning synthesized substances can be found in the Supplementary material.

2.1.1. Protection of amino groups via carbonodithioimidic acid dimethyl ester

A solution of 2-(dimethylamino)ethylamine (**1**) (8.82 g, 0.1 mol) and triethylamine (TEA, 20.2 g, 0.2 mol) in dry $CHCl_3$ (75 ml) was stirred at room temperature. Carbon disulfide (8.36 g, 0.11 mol)

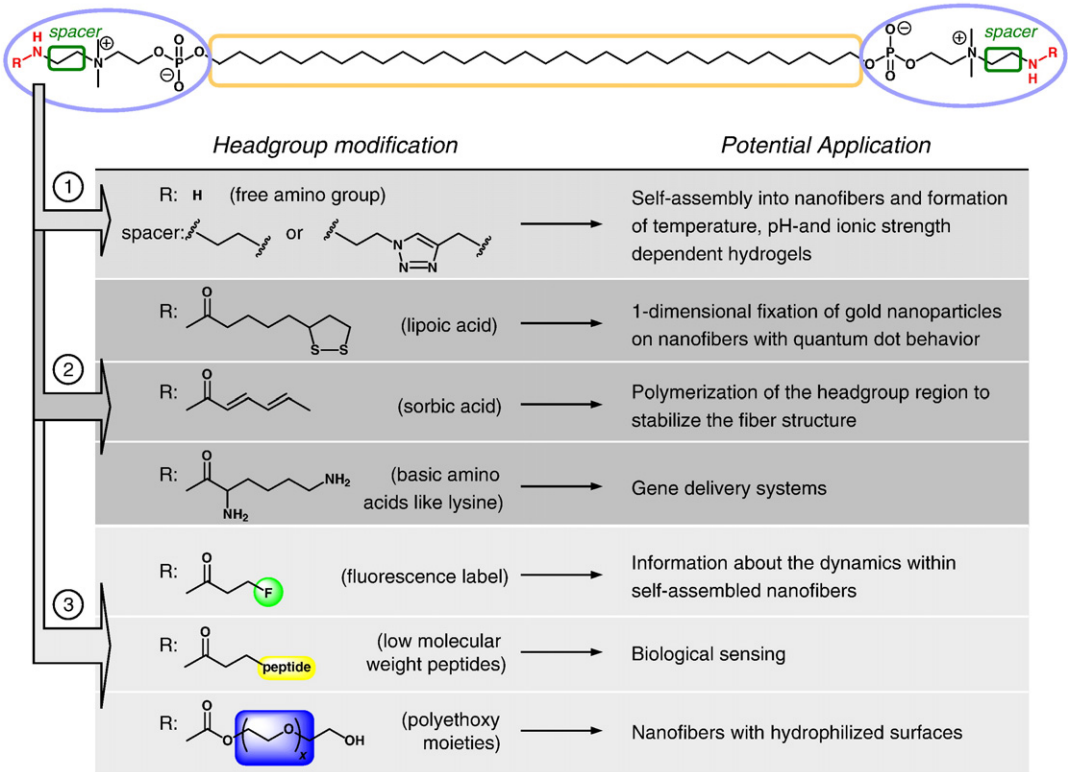


Fig. 1. Potential applications of amino-functionalized bolalipids with different headgroup modifications: Arrow 1 points to amino-functionalized bolalipids, arrow 2 shows headgroup modifications with carboxylic acids described in the manuscript, and arrow 3 indicates potential modifications that are currently under investigation.

dissolved in dry $CHCl_3$ (20 ml) was added in such a rate that the temperature remained below 50 °C and stirring was continued for further 1 h. Afterwards, methyl iodide (15.6 g, 0.11 mol) was added slowly and the mixture was heated at reflux for 3 h. After cooling down to room temperature, the organic layer was washed with water (2×150 ml) and concentrated to dryness under reduced pressure. The residue was dissolved again in Et_2O (250 ml), washed with water (2×50 ml), dried over sodium sulfate, and concentrated to dryness under reduced pressure. The obtained light yellow crystals of [2-(dimethylamino)ethyl]carbamodithioic acid methyl ester (**2**) (12.33 g, 69.2 mmol, 69% yield) was dried in vacuo over phosphorus pentoxide. For the second methylation, **2** was dissolved in dry acetone (50 ml) under argon atmosphere, a solution of sodium bis(trimethylsilyl)amide in THF (1 M, 70 ml) was added slowly, and the mixture was stirred for 2 h. Afterwards, methyl iodide (9.94 g, 70 mmol) was added and the resulted mixture was heated at reflux for further 2 h. For work-up the solvent was evaporated and the residue was dissolved in Et_2O (100 ml), washed with water (2×30 ml), dried over sodium sulfate concentrated to dryness under reduced pressure to give the [2-(dimethylamino)-ethyl]carbonodithioimidic acid dimethyl ester (**3**) as oily substance in 90% yield (11.98 g, 62.3 mmol) and 62% over-all yield.

2.1.2. Phosphorylation and quarternisation

β-Bromoethylphosphoric acid dichloride [18] (1.93 g, 8 mmol) was poured into dry $CHCl_3$ (20 ml) under cooling with ice/water. A mixture of dry TEA (1.42 g, 14 mmol) and dry $CHCl_3$ (20 ml) was added slowly and stirring was continued for 30 min at 0 °C. Dotriacontane-1,32-diol (**4**) [7] (0.48 g, 1 mmol) was added as solid substance in one portion. The suspension was heated to 60 °C until diol **4** was dissolved, then rapidly cooled to room temperature. Stirring was continued for further 24 h at this temperature. After TLC control ($CHCl_3/Et_2O$, 8/2, v/v) showed complete conversion of diol **4**, crushed ice (40 ml) was added and the mixture was stirred for a further 2 h. The organic layer was separated and the aqueous phase was diluted with cold saturated solution of NaCl (50 ml) and then extracted with $CHCl_3$ (3×50 ml). The combined organic phases were concentrated to dryness under reduced pressure, the oily residue was dissolved in THF/water (9/1, v/v, 30 ml) and stirred for 1 h. Then, the solvent was evaporated and the oily residue was transferred into a mixture of dry $CHCl_3$ (30 ml), dry acetonitrile (30 ml) and dry EtOH (10 ml) under argon atmosphere. The amine **3** (7.7 g, 40 mmol) was added and the mixture was kept in a closed tube at 45 °C for 72 h. Afterwards, the mixture was concentrated by evaporation of the solvent and the residue was purified by MPLC using gradient technique and $CHCl_3$/MeOH/water as eluent to give the dotriacontane-1,32-diyl-bis{2-[*N*-(2-{[bis(methylthio)methylen]amino}ethyl)-*N*,*N*-dimethylammonio]ethylphosphate} (**5**) as white solid (42% yield) which was dried in vacuo over phosphorus pentoxide at room temperature for at least two days.

2.1.3. Deprotection of amino groups — synthesis of the amino-functionalized bolalipid

The bolalipid **5** (26.8 mg, 24.8 μmol) was suspended in water (10 ml) under slightly warming, 0.2 ml HCl (2 M) was added and the mixture was stirred at room temperature. After 24 h, the mixture was alkalized with a solution of potassium carbonate (pH ~ 10) and evaporated under reduced pressure until a small amount of a clear colorless solution is left. Addition of dry acetone resulted in the precipitation of dotriacontane-1,32-diyl-bis{2-[*N*-(2-aminoethyl)-*N*,*N*-dimethylammonio]ethylphosphate} (AEPC-C32-AEPC, **6**) as white solid. The product was collected by centrifugation and was dried in vacuo over phosphorus pentoxide at room temperature.

2.1.4. Synthesis of 2-azidoethylamine

2-Bromoethylamine hydrobromide **7** (1.0 g, 4.9 mmol) was added to a solution of sodium azide (0.95 g, 14.6 mmol) in water (5 ml). The stirred solution was heated to 80 °C for at least 24 h before it was cooled down to 0 °C. Et_2O (5 ml) and subsequent solid KOH (1.6 g) were added. After vigorous stirring for 30 min, the organic phase was separated and the aqueous layer was extracted with Et_2O (3×25 ml). The combined organic layers were dried over sodium sulfate, filtered, and the solvent was removed carefully under reduced pressure affording the azide **8** as a light yellow liquid which was used without further purification. ^{1}H NMR spectra were found to be identical with the one described in the literature [19].

2.1.5. General procedure for synthesis of amides from 2-azidoethylamine

Equimolar amounts of 2-azidoethylamine **8** (86 mg, 1 mmol), the appropriate carboxylic acid, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 442 mg, 1 mmol), and 1.5fold excess of TEA (150 mg, 1.5 mmol) were dissolved in dry CH_2Cl_2 (10 ml) and the mixture was stirred at room temperature. After 24 h, the solvent was removed under reduced pressure and the residue was purified by MPLC using gradient technique and heptane/$CHCl_3$ or $CHCl_3$/MeOH, respectively, as eluent affording the carboxylic acid *N*-(2-azidoethyl)amides **9a–9c** in 80–91% yield.

2.1.6. Coupling of carboxylic acid N-(2-azidoethyl)amides with propynyl modified bolalipid by click-reaction

The *N*-(2-azidoethyl)carboxylic acid amides **9a–9c** as well as the azide **8** were used for subsequent, copper-catalyzed click-reaction with the propynyl modified phosphocholine dotriacontane-1,32-diyl-bis[2-(*N*,*N*-dimethyl-*N*-propynylammonio)ethylphosphate] (**10**) described previously [13]. Propynylcholine **10** (10 mg, 11.6 μmol) and the appropriate azide **8** or **9a–9c**, respectively, (30 μmol) were suspended in water/EtOH (10 ml, 2/1, v/v). Sodium ascorbate (8 μmol in aqueous solution) followed by copper(II) acetate monohydrate (4 μmol in aqueous solution) were added and the mixture was stirred for 24 h at room temperature. After evaporation of the solvent, the residue was purified by column chromatography using gradient technique and $CHCl_3$/MeOH/water as eluent affording functionalized bolalipids **11a–11c** and the amino group containing bolalipid **11d** (AETPC-C32-AETPC).

2.1.7. Cleavage of BOC-protecting groups

For cleavage of the BOC-protecting groups, the bolalipid **11c** (10 mg, 5.9 μmol) and equimolar amounts of trifluoroacetic acid (TFA) were dissolved in dry CH_2Cl_2 (10 ml) and stirred for at least 48 h at room temperature. After evaporation of the solvent, the residue was purified by MPLC using gradient technique and $CHCl_3$/MeOH/water as eluent.

2.2. Sample preparation

The appropriate amount of the pure bolalipid was suspended in water (pH 6) or 10 mM carbonate buffer at pH 10 for DSC and TEM measurements. Binary bolalipid mixtures were prepared from lipid stock solution in $CHCl_3$/MeOH (2/1, v/v) as solvent by mixing of appropriate volumes of the stock solution. The organic solvent was then rapidly removed in a stream of nitrogen at elevated temperatures. The resulting bolalipid films were kept in an evacuated flask for 24 h to remove residual traces of solvent. The aqueous suspensions were then prepared by adding a certain volume of deionised water and 1 M NaCl solution, respectively. Homogenous suspensions were obtained by heating to 90 °C and vortexing the sample.

2.3. DSC

DSC measurements were performed using a MicroCal VP-DSC differential scanning calorimeter (MicroCal Inc. Northampton, MA, USA). Before the measurements, the sample suspension (1 mg/ml) and the water or buffer reference were degassed under vacuum while stirring. A heating rate of 20 K/h was used, and the measurements were performed in the temperature interval from 2 to 95 °C. To check the reproducibility, three consecutive scans were recorded for each sample. The buffer–buffer

baseline was subtracted from the thermograms of the samples, and the DSC scans were evaluated using MicroCal Origin 7.0 software.

2.4. TEM

The negatively stained samples were prepared at 5 °C by spreading 5 µl of the dispersion onto a Cu grid coated with a formvar-film. After 1 min, excess liquid was blotted off with filter paper and 5 µl of 1% aqueous uranyl acetate solution were placed onto the grid and drained off after 1 min. The dried specimens were examined with a Zeiss EM 900 transmission electron microscope.

2.5. DLS

The DLS experiments were performed with an ALV-NIBS-HPPS particle sizer (ALV-Laser Vertriebsgesellschaft mbH, Langen, Germany). The device was equipped with a 3 mW HeNe laser with a wavelength of 632.8 nm. Because of the principle of noninvasive back scattering the scattering angle was 173°. All samples (1 mg/ml) were freshly prepared and then filtered through a membrane filter of 0.2 µm pore size (at 80 °C) into quartz cuvettes (HELLMA GmbH & Co. KG, Muehlheim, Germany). Before starting the measurement, each sample was equilibrated for 60 min. Three individual measurements were performed for each system to test the reproducibility. The experimental data were analyzed with the aid of the ALV-5000/E software based on the modified CONTIN method [20], with the temperature correction of the viscosity being taken into account.

2.6. Rheology

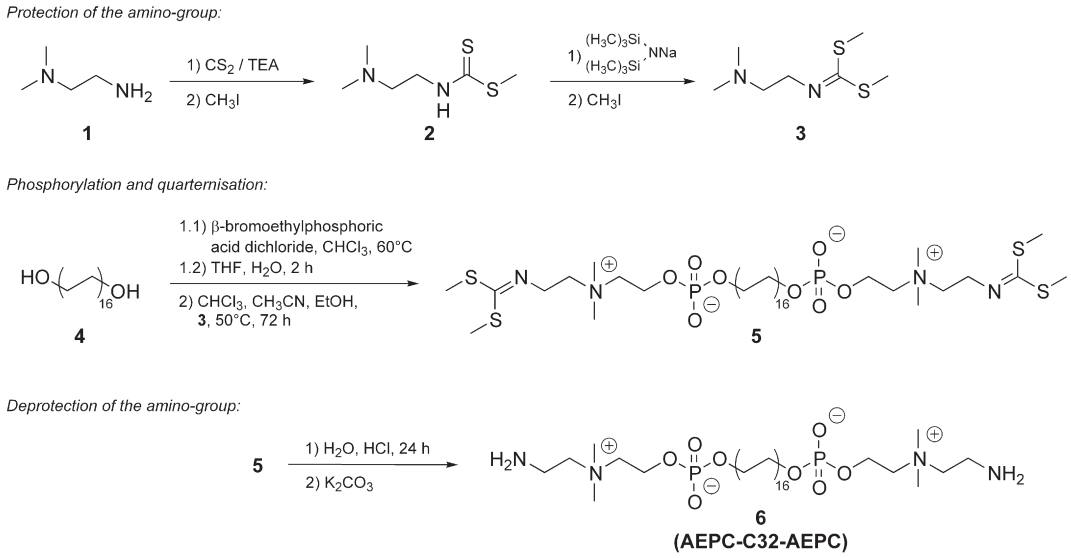
Rheological properties of 1 mg/ml bolalipid suspensions in water were studied using a MCR301 rheometer (Anton Paar, Germany) controlled by the software Rheoplus 3.0. For the oscillatory measurements we used a cone and plate shear geometry (2°/50 mm) with a thermostatting unit (Peltier system: −40 to 200 °C).

3. Results and discussion

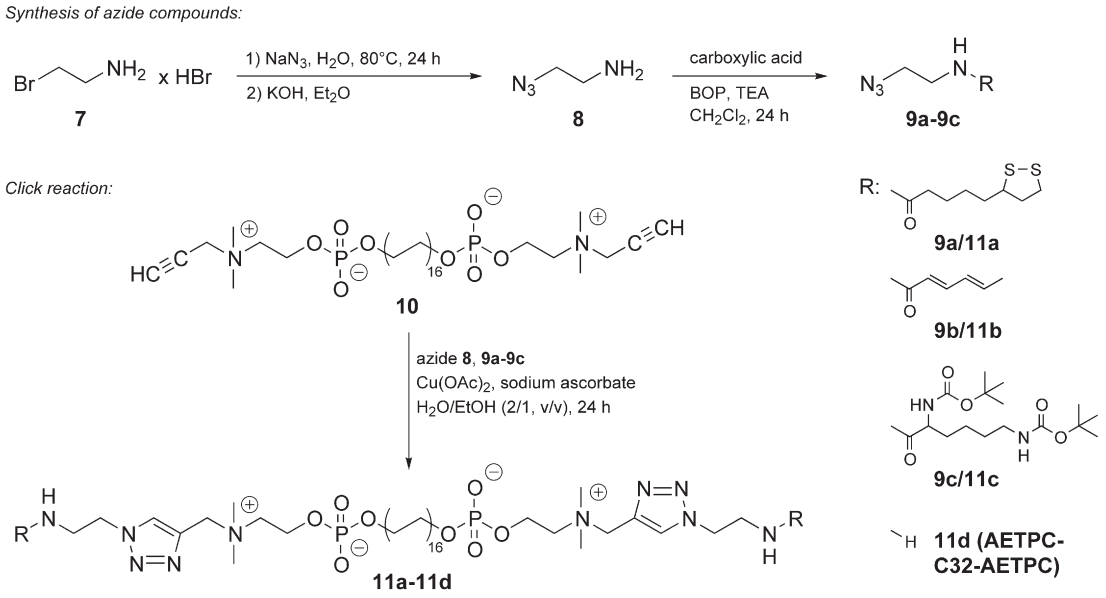
3.1. Synthetic methods

For the synthesis of symmetrical, amino-functionalized single-chain bolalipids we established two different strategies. The first approach uses the well-established bis-phosphorylation of long-chain 1,ω-diols and subsequent quarternisation with amines [7]. As we described recently, the use of 2-(dimethylamino)ethylamine (**1**) during the quarternisation reaction resulted in a mixture of secondary and quarternary amines [13]. A separation and purification of single compounds was not possible due to the very similar chromatographic properties of the products obtained. To avoid the problem of two reaction centers within the amine **1** the primary amino group had to be protected as imine (Schiffs base) using various aldehydes or ketones. Considering that an increase of the amine size led to lower yields during the quarternisation [13] common protecting groups for primary amino moieties such as *tert.*-butoxycarbonyl (Boc) [21], benzyloxycarbonyl (Cbz) [22] or 9-fluorenylmethyloxycarbonyl (Fmoc) [23] should be evaded. The first attempt employing acetone as protecting reagent failed because of polymerization and decomposition of the reaction mixture. A second attempt dealt with carbon disulfide in combination with a two-step alkylation with methyl iodide. In contrast to the procedures described by SAUTER et al. [24] potassium carbonate used for the second deprotonation of [2-(dimethylamino)ethyl]carbamodithioic acid methyl ester (**2**) had to be replaced by sodium bis(trimethylsilyl)amide in order to obtain acceptable yields (62% over-all yield) of the [2-(dimethylamino)-ethyl]carbonodithioimidic acid dimethyl ester (**3**). The one-side protected amine **3** was then used for the quarternisation of the phosphorylated dotriacontane-1,32-diol (**4**). For the foregoing phosphorylation reaction we used β-bromoethylphosphoric acid dichloride in combination with TEA as basic additive owing to the higher efficiency of this reagent compared to 2-chloro-1,3,2-dioxophospholane or phosphites which were also used by other groups performing bolalipid synthesis [25,26]. This two-step procedure resulted in the formation of dotriacontane-1,32-diyl-bis{2-[*N*-(2-{[bis(methylthio)methylen]amino}ethyl)-*N*,*N*-dimethylammonio] ethylphosphate} (**5**) in moderate yields (42%) with regard to the diol **4**. For the cleavage of the imidic protecting group the bolalipid **5** was suspended in diluted hydrochloric acid and stirred of 24 h affording the amino-functionalized bolalipid dotriacontan-1,32-diyl-bis{2-[*N*-(2-aminoethyl)-*N*,*N*-dimethylammonio]ethylphosphate} (AEPC-C32-AEPC, **6**) in nearly quantitative yields. The complete synthesis of AEPC-C32-AEPC (**6**) is summarized in Scheme 1.

The deprotonation of the primary amino groups of AEPC-C32-AEPC (**6**) could be realized by addition of an aqueous solution of potassium carbonate. The influence of the degree of protonation on the temperature-dependent aggregation behavior of AEPC-C32-AEPC (**6**) is explained in Section 3.2.



Scheme 1. Synthesis of the amino-functionalized bolalipid **6** (AEPC-C32-AEPC) via phosphorylation and quarternisation reaction.



Scheme 2. Synthesis of headgroup modified bolalipids **11a–11d** using *click*-reaction in combination with 2-azidoethylamides.

For the second synthetic approach of amino-functionalized single-chain bolalipids we used a *click*-reaction [27,28] of propynes with azides whose feasibility was shown previously [13]. The advantages of this copper(I) catalyzed *Huisgen* 1,3-cyclo addition are 1) the modular and broad applicability, 2) simple reaction procedures at ambient temperatures, and 3) high yields avoiding the formation of by-products. Nevertheless, the reaction might be limited to the marginal commercial availability of functionalized azides. To overcome this restriction, the use of 2-azidoethylamine as linker was proposed. Therefore, 2-bromoethylamine hydrobromide (**7**) was converted into the amine **8** using sodium azide proceeding as described by MAYER et al. [19] and, afterwards, this amine **8** was coupled with various carboxylic acids deploying (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) as the coupling reagent. This reaction procedure led to the formation of *N*-(2-azidoethyl)amides of 1,2-dithiolan-3-pentanoic acid (α-lipoic acid, **9a**), hexa-2(*E*),4(*E*)-dienoic acid (sorbic acid, **9b**), and 2,6-bis(*tert.*-butoxycarbonylamino)hexanioc acid (di-BOC-lysine, **9c**). The purification of the final products was carried out using MPLC with a gradient technique and heptane/$CHCl_3$ or $CHCl_3$/MeOH, respectively, as eluent affording the azidoamides **9a–9c** in 80 – 91% isolated yields (see Scheme 2).

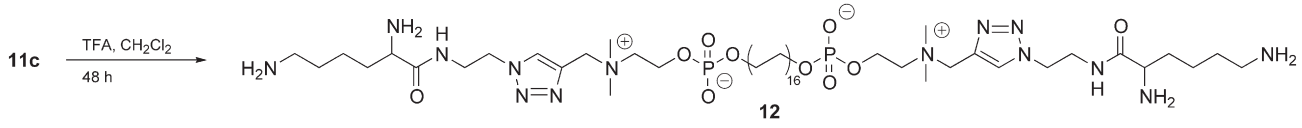
The azidoamides **9a–9c** were used for subsequent copper(I) catalyzed *click*-reaction with the propynyl modified bolalipid dotriacontane-1,32-diyl-bis[2-(*N*,*N*-dimethyl-*N*-propynylammonio)ethylphosphate] (**10**) and catalytic amounts of copper(I) salt which was prepared in situ by reduction of copper(II) acetate with sodium ascorbate and water/EtOH (2/1, v/v) as solvent [13,28]. After 24 h, thin-layer chromatography (TLC) indicated the complete conversion of the propynylcholine **10** into headgroup modified bolalipids containing α-lipoic acid (**11a**), sorbic acid (**11b**), and di-BOC-lysine (**11c**), respectively (see Scheme 2). In addition, the unmodified 2-azidoethylamine (**8**) was also used for *click*-reaction resulting in the formation of dotriacontane-1,32-diyl-bis[2-(*N*-{[1-(2-aminoethyl)-1,2,3-triazol-4-yl]methyl}-*N*,*N*-dimethylammonio)ethylphosphate] (AETPC-C32-AETPC, **11d**). The AETPC-C32-AETPC represents the second amino-functionalized bolalipid introduced in this work. It's temperature-dependent aggregation behavior is described in Section 3.2. The physico-chemical characterization of the headgroup modified bolalipids **11a–11c** is currently under investigation.

For the synthesis of the lysine modified bolalipid **12** the BOC-protecting groups were cleaved using trifluoroacetic acid (TFA). Therefore, the bolalipid **11c** was suspended in CH_2Cl_2 with equimolar amounts of TFA and stirred for at least 48 h at room temperature resulting in the dotriacontane-1,32-diyl-bis(2-{*N*-[(1-{2-[(2,6-diamino-1-oxohex-1-yl)amino]ethyl}-1,2,3-triazol-4-yl)methyl]-*N*,*N*-dimethylammonio}ethylphosphate) (**12**) (see Scheme 3).

3.2. Temperature-dependent aggregation at different pH values

The self-assembly of symmetric single-chain bolalipids in water is driven by hydrophobic, electrostatic, and hydrogen bonding interactions. In addition, the ratio between the cross-sectional area of the chain and the headgroup of the bolalipid strongly determines the shape of the formed aggregates. In the case of the amino-functionalized bolalipid AEPC-C32-AEPC each headgroup is composed of a phosphate group and an *N*-(aminoethyl)-*N*,*N*-dimethylammonium group with a permanent positive charge at the quarternary nitrogen (the *N*,*N*-dimethylammonium part) and a dissociable proton at the primary nitrogen (the aminoethyl part). Therefore, at intermediate pH the bolalipid molecule should be positively charged due to the protonation of the aminoethyl group, whereas at higher pH the dissociation of this proton should lead to a lipid with zwitterionic headgroups (see Fig. 2).

At very low pH-values the headgroup should be doubly positively charged due to the protonation of the phosphate and the aminoethyl group. Recently, we determined the pK_a values of a similar bolalipid with phosphodimethylammonium groups by titration in water [11]. The measurements yielded an apparent pK_a value for the phosphate group of 3.3, and one of 6.5 for the dimethylammonium group.



Scheme 3. Synthesis of the lysine modified bolalipid **12**.

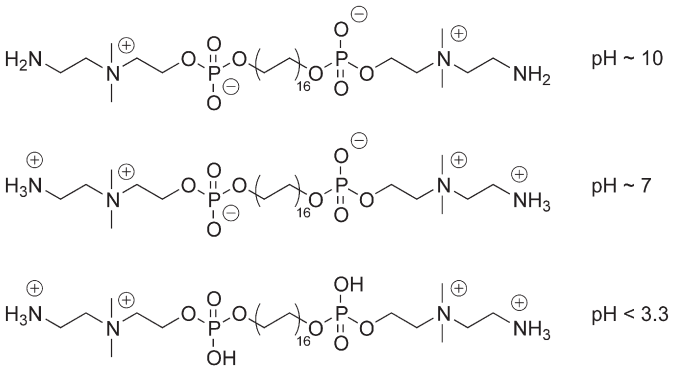


Fig. 2. Headgroup protonation state of the bolalipid AEPC-C32-AEPC at different pH values.

According to these results and based on pK_a values of basic amino acids [29], the investigations with AEPC-C32-AEPC and AETPC-C32-AETPC were carried out either in water at pH 6, where the headgroups are positively charged, or in carbonate buffered solutions at pH 10, where the bolalipids has zwitterionic headgroups.

The temperature-dependent aggregation behavior of AEPC-C32-AEPC and AETPC-C32-AETPC was investigated by transmission electron microscopy (TEM), differential scanning calorimetry (DSC) and dynamic light scattering (DLS). Fig. 3 shows TEM images of stained (uranyl acetate) suspensions of both bolalipids at pH 6 and 10 that were prepared at 5 °C. The suspensions at pH 6 indicate the formation of short fiber aggregates with a diameter of about 5 nm that corresponds roughly to the molecular length of the bolalipids. AEPC-C32-AEPC forms a few long fibers (1 μm) but mainly short fiber segments with up to 50 nm length are visible.

In contrast, the fibers composed of AETPC-C32-AETPC are up to 300 nm long and they arrange in a parallel fashion. At pH 10, both bolalipids self-assemble into long and flexible fibers with a length of more than 1 μm.

To clarify the temperature-dependent stability of these nanofibers, we performed DLS measurements at temperatures that were chosen according to the transition temperatures detected by DSC measurements. Fig. 4 shows the DSC curves for suspensions of both amino-functionalized bolalipids at pH 6 and 10, respectively. All heating curves display two endothermic transitions. The fibers visualized by TEM are present up to the first transition temperature T_m1. Above this temperature, DLS measurements indicate the presence of spherical aggregates with a diameter of about 4 to 7.6 nm (see Supplementary material). In Table 1, the DSC transition temperatures T_m1 and T_m2 are summarized together with the radii of micelles that were determined by DLS at temperatures between T_m1 and T_m2 as well as above T_m2. The transition temperatures T_m1 of AEPC-C32-AEPC and AETPC-C32-AETPC are more than 15 K lower for the suspensions at pH 6 than for pH 10. In addition, all transitions are very broad, which indicates that they are less cooperative.

All heating curves show a very broad second transition (T_m2), which occurs within the stability range of the micellar aggregates (see Table 1). The cooling curves indicate almost no hysteresis for the suspensions at pH 6 but a strong hysteresis for pH 10. These distinct differences for both bolalipids at pH 6 and pH 10 should be due to differences in the protonation state of the headgroups. At pH 6, the positively charged headgroups cause repulsive interactions between neighboring headgroups and, as a consequence, the T_m values are significantly lower.

We were interested in the gelling properties of symmetric long-chain bolalipids that originate from entangled fiber aggregates. For some of these bolalipids the formation of hydrogels has been

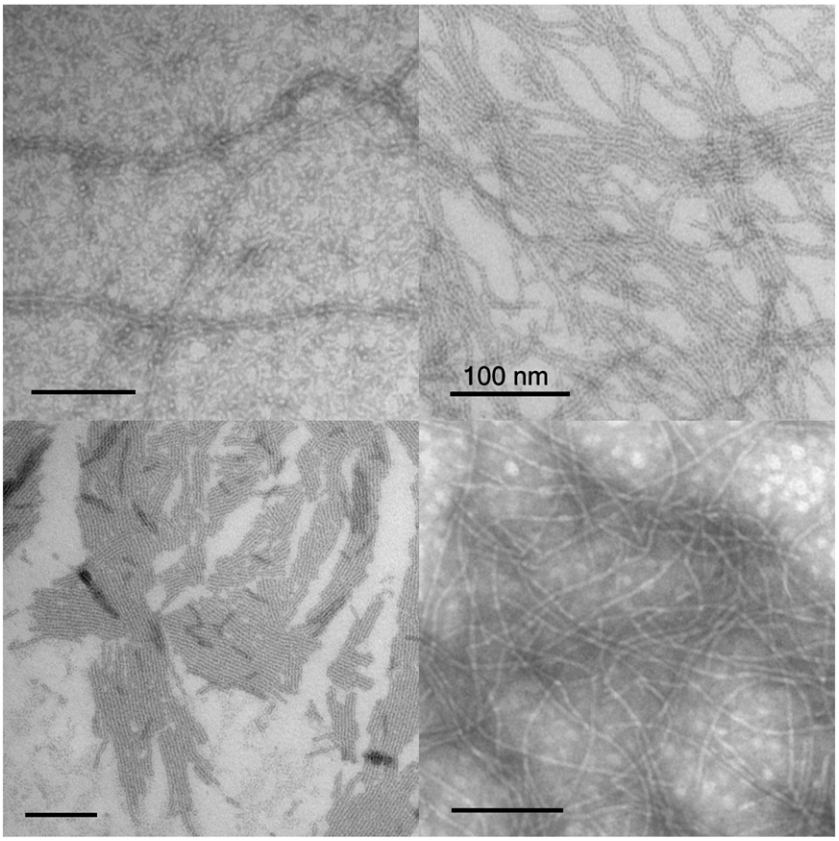


Fig. 3. TEM images of stained (uranyl acetate) suspensions (0.1 mg/ml) of amino-functionalized bolalipids. Top row: AEPC-C32-AEPC at pH 6 (left) and pH 10 (right), Bottom row: AETPC-C32-AETPC at pH 6 (left) and pH 10 (right). The bar corresponds to 100 nm.

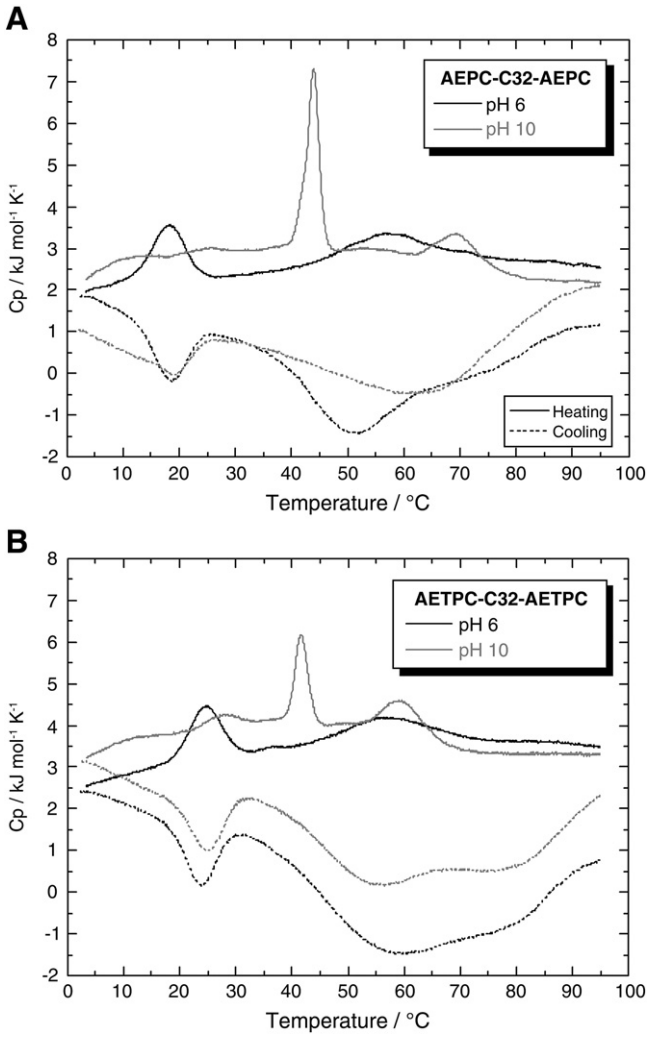


Fig. 4. DSC heating curves of AEPC-C32-AEPC (A) and AETPC-C32-AETPC (B) at pH 6 and 10, respectively. Cooling curves are presented with dashed lines.

described recently [9,30–33]. The fibers become cross-linked by noncovalent interactions so that they are able to trap water molecules. As a consequence, the hydrogels are often thermally reversible and the gel properties can easily be controlled by changing the temperature, pH, or ionic strength of the suspension.

The bolalipids AEPC-C32-AEPC and AETPC-C32-AETPC in dilute aqueous suspension (1 mg/ml) show no pronounced gelling properties. This can be explained by the absence of a fiber network and the presence of short fiber segments at pH 6. In contrast, the phosphocholine analogue PC-C32-PC exhibits excellent properties as a hydrogelator at room temperature due to the formation of a dense network of cross-linked fibers [9]. However, a 1:10 mixture of AEPC-C32-AEPC and PC-C32-PC in the presence of NaCl (1 M) shows the formation of a viscous, transparent gel, which is stable up to temperatures of about 48 °C. The gel state is due to the existence of a fiber network. Salt was added to screen the positive headgroup charges of AEPC-C32-AEPC at pH 6 and to prevent repulsive interactions between neighboring headgroups. DSC measurements indicate the transformation of fibers into spherical micelles at 48 °C and they show that 1 M NaCl has only a marginal effect onto the transition temperatures (see Supplementary material). Rheograms show at 2, 20, and 60 °C the typical signature of a viscoelastic fluid (not shown). At low frequencies, both storage and loss moduli are rather independent of frequency, and at 2 and 20 °C G' is about an order of magnitude larger than G''. Similar values for G' and G'' were found for PC-C32-PC at 2 and 20 °C. Above the first transition temperature at 48 °C, the shear modulus G' breaks down (see Fig. 5A) and the gel character is lost due to the transformation of fibers into spherical micelles.

After continuous heating of the samples up to 80 °C and subsequent cooling to 2 °C, the moduli are significantly lower than before the temperature treatment. However, at 2 °C both moduli show a distinct increase during the next 10 h. Whereas the pure PC-

Table 1
DSC transition temperatures (T_m1 and T_m2) of aqueous suspensions (1 mg/ml) of AEPC-C32-AEPC and AETPC-C32-AETPC, as well as radii of micelles determined by DLS at temperatures between T_m1 and T_m2 and above T_m2 at pH 6 and 10, respectively.

Bolalipid	pH	DSC		DLS	
			T_m [°C]	T [°C]	Radius [nm]
AEPC-C32-AEPC	6	T_m1	18.2	30	2.0 ± 0.1
		T_m2	57.0	75	1.8 ± 0.1
	10	T_m1	44.0	55	3.8 ± 0.1
		T_m2	70.0	80	3.2 ± 0.1
AETPC-C32-AETPC	6	T_m1	24.6	35	3.1 ± 0.1
		T_m2	56.0	75	2.7 ± 0.1
	10	T_m1	41.6	50	3.3 ± 0.1
		T_m2	59.0	75	3.1 ± 0.1

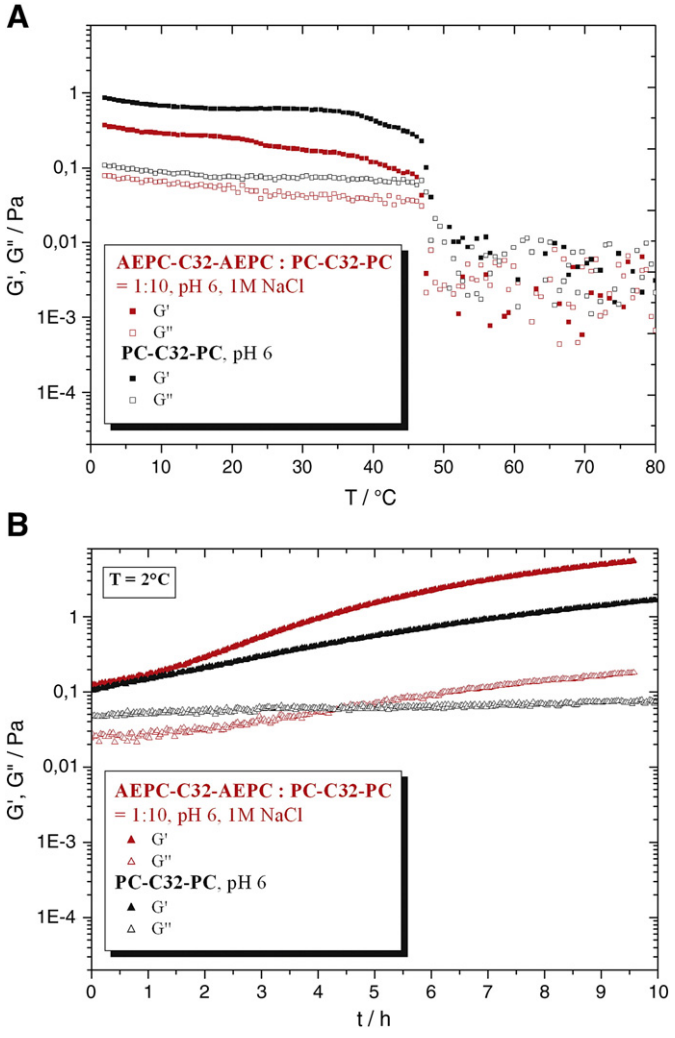


Fig. 5. (A) Temperature dependence of G' and G'' of aqueous suspensions (1 mg/ml) of a 1:10 AEPC-C32-AEPC/PC-C32-PC mixture in the presence of 1 M NaCl recorded at a deformation γ of 1% and a frequency ω of 1 rad/s. Moduli for pure PC-C32-PC are added for comparison. The heating rate was 20 K/h. (B) Time dependence of G' and G'' at 2 °C after heating the sample to 80 °C and subsequent cooling to 2 °C. The heating and cooling rate was 20 K/h.

C32-PC suspension reaches the initial G' and G'' values, the AEPC-C32-AEPC/PC-C32-PC mixture (1:10) shows a large increase of both moduli of more than one order of magnitude (see Fig. 5B). This is probably due to the formation of intermolecular hydrogen bonds between headgroups of AEPC-C32-AEPC. In contrast, PC-C32-PC is not able to form intermolecular hydrogen bonds. These results clearly indicate that a tailor-made modification of the bolalipid headgroup is a promising way to enhance the gelling properties of bolalipid hydrogelators.

4. Conclusion

In summary, we developed the synthesis of two novel, amino-functionalized bolalipids (AEPC-C32-AEPC and AETPC-C32-AETPC) using either a one-side protected diamine for the quarternisation reaction of the phosphorylated dotriacontane-1,32-diol or the *click*-chemistry in combination with a 2-azidoethylamine. Especially the second way offers the possibility to fix versatile carboxylic acids, e.g. α-lipoic acid, sorbic acid, or lysine, leading to different tools for the use in biochemical and biophysical sensing. Actually, the insertion of a fluorescence label is under investigation. This label will be used to get information about the dynamics of the bolalipid molecules within the self-assembled nanofibers. The insertion of the additional amino-moiety within the bolalipid headgroup gives rise to a pH-dependent adjustment of the self-assembly properties ranging from short fiber segments at pH 6 to long and flexible fibers at pH 10. Furthermore, the addition of AEPC-C32-AEPC to a hydrogel composed of unmodified PC-C32-PC can enhance the gelling properties of bolaform hydrogelators.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2010.01.013.

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