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Template-free Self-assembling Fullerene and Lipopeptide Conjugates of Alamethicin Form Voltage-Dependent Ion Channels of Remarkable Stability and Activity‡

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Abstract: *N*- and *C*-terminally modified with fullerene or lipopeptide alamethicin molecules were designed for the formation of template-free, self-assembling, voltage-dependent ion conducting channels. The automated solid phase synthesis of the alamethicin-F30 sequence was performed by *in situ* fluoride activation on 2-chlorotritylchloride-polystyrene resin and the conjugation with fullerenes-C_{60} and -C_{70} was carried out in solution. Voltage-dependent bilayer experiments revealed preferred channel sizes for *C*-terminal alamethicin F30-fullerene-C_{60} and -C_{70} conjugates and higher activity compared with native alamethicin, whereas *N*-terminally linked fullerene balls destabilize pore formation. *C*-terminal alamethicin F30-fullerene-C70 conjugates show pore states with remarkably long lifetimes of seconds. *C*-terminal lipopeptide conjugates of alamethicin were prepared by coupling via short peptide spacers with synthetic tripalmitoyl-S-glyceryl-cysteine, which represents the strong membrane anchoring *N*-terminus of bacterial lipoprotein. Alamethicin-lipopeptide conjugates exhibit high channel forming activities, whereby they self-assemble and adopt preferred pore states with extremely long lifetimes. The novel membrane modifying peptaibol constructs are valuable lead compounds for developments in sensorics related to transmembrane ion conductance.
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Keywords: alamethicin; peptaibols; fullerene; lipopeptides; ion channels; lipid bilayer membranes

INTRODUCTION

Biophysical interest in alamethicin is due to its potential dependent ion conducting properties in black lipid membranes. The ion channels are caused by a supramolecular system of several amphiphilic α-helices forming fluctuating pores of different sizes with discrete conductance states [1]. Therefore, alamethicin and related membrane spanning α-helical polypeptides belong to model systems [2,3] for ligand and voltage driven channel proteins which play a central role in signal transduction through membrane barriers. Biophysical investigations of peptaibol analogues with properties designed for channel formation [4–7] revealed new insights into the biological functionalities of defined structural elements such as helix/helix interactions or charge effects in transmembrane proteins.

Abbreviations: Aca, ε-aminocaproic acid; Aib, α-aminoisobutyric acid; BLM, black lipid membrane; CIS-MS, coordination ion spray-mass spectrometry; Pheol, L-phenylalaninol; TFFH, tetramethylfluoroformamidinium hexafluorophosphate; Pam_3Cys, tripalmitoyl-S-glyceryl-L-cysteine.

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Peptaibol isolates from microorganisms represent often highly complex natural peptide libraries [8] but all prominent alamethicin-like peptaibols possess an amphiphilic α-helix with a high dipole moment [9–11] and a proline bend, conformational restriction and protease resistance due to the high content of α-aminoisobutyryl residues [12–14]. In order to avoid the natural microheterogeneous mixtures and to provide analytically well defined alamethicin preparations of the highest purity for biophysical studies alamethicin was synthesized first by solution phase segment condensations [15] and later by automated solid phase peptide synthesis using *in situ* fluoride activation [16].

Based on the biophysical and structural examination of a large number of synthetic, systematically varied analogues of alamethicin the flip-flop gating model [11] has been proposed, which is an extension of the barrel stave model [1] and explains the dynamics and potential dependence of the pore forming process. The occurrence of defined conductance states of self-assembled peptaibol molecules is proposed to depend on the presence of the amino acids Gln7, Gly11, Pro14 (QGP structural motif) of the alamethicin sequence, whereby Gln7 contributes hydrogen bridges between helix monomers, thus stabilizing preferred aggregates.

Interestingly, ion conducting pore formation for all members of the sequential peptide oligomers Boc-(Ala-Aib-Ala-Aib-Ala)$_n$-OMe (n = 1–4) was found [4]. For most of such artificial ion conductors no resolved single pore states can be recorded except for dansyl-(Ala-Aib-Ala-Aib-Ala)$_3$-Pro-Ala-Aib-Ala-Aib-Ala-Trp-OMe [17], for example, which exhibits the channel characteristics of the acetylcholine receptor. The latter peptide is a typical example of pore state preferences introduced by chemical end group modifications of Aib containing peptide helices.

In the context of *C*-terminally modified natural alamethicins a particular property of the negatively charged semisynthetic alamethicin-F50-phosphate [7] should be mentioned. By addition of polycationic polypeptides voltage dependent single ion channels formed by alamethicin-phosphate could be transiently blocked. Similar effects were obtained with *C*-terminally biotinylated natural alamethicin-F50 which upon addition of streptavidin shows a strong prolongation of defined pore states. The molecular dynamics of α-helical membrane spanning channel formers have been studied on a variety of *C*-terminally fluorophore- or spin-labelled alamethicin-like polypeptides using time resolved spectroscopic methods [5,6,18,19].

Here, two alamethicin conjugates are focused on, both of which were designed to exhibit a prolonged lifetime and preference of their single pore states and in addition a strong asymmetry of the ion conductance in lipid bilayer membranes. Moreover, the constructs were designed for higher membrane activity, e.g. the induction of pore formation at a lower concentration than alamethicin. To realize this idea fullerenes were coupled at the *N*- or *C*-terminus of synthetic alamethicin-F30 in order to obtain helix-and-ball conjugates and lipopeptides were coupled to the *C*-terminus of natural alamethicin-F50 to obtain helix-and-membrane anchor conjugates.

MATERIALS AND METHODS

Reagents and Peptide Synthesis

Fmoc-amino acids, natural alamethicin, lipopeptide and reagents. All Fmoc-amino acids and 2-chlorotritylchloride-polystyrene resin were from NovaBiochem (Bad Soden, Germany). Most reagents, fine chemicals and solvents were from Fluka (Neu-Ulm, Germany) or Merck (Darmstadt, Germany). Natural alamethicin F50 was isolated from the culture medium of *Trichoderma viride* NRRL 3199 [7]. The synthetic lipoamino acid N-palmitoyl-S-[2,3-(bispalmitoyloxy)propyl]-cysteine (Pam_3Cys) [20] and lipopeptides were from EMC microcollections GmbH (Tübingen, Germany). Fullerene C_{60}- and C_{70}-carboxylic acid derivatives were prepared as described [21].

Tetramethylfluoroformamidinium hexafluorophosphate (TFFH). The coupling reagent TFFH was described by Carpino *et al.* [22,23] and is recommended for peptide synthesis with α,α-dialkylated amino acids [16,24] but is not useful for N-alkylated amino acids [25]. A simple one-pot synthesis protocol was formed as follows. Oxalyl chloride (10 ml, 0.11 mol) was added dropwise to tetramethylurea (12 ml, 0.1 mol) in water-free dichloromethane (50 ml) in a dry N_2 atmosphere, whereby immediate development of CO_2 and CO occurred. After refluxing for 2 h and removal of solvents and excess of oxalylchloride in high vacuum, the residual tetramethylchloroformamidinium chloride was dissolved immediately in dry acetonitrile in a N_2 atmosphere. Potassium hexafluorophosphate (20.24 g, 0.11 mmol; dried at 120 °C *in vacuo* for 16 h

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and stored at 100 °C), dried potassium fluoride (17.5 g, 0.3 mol) and dibenzo-18-crown-6 (120 mg) were added. After stirring for 16 h precipitated KCl was filtered off and washed with acetonitrile. The solution was evaporated almost to dryness and diethylether was added. The product was recrystallized from acetonitrile/diethylether. Yield 21 g (89%); ^{1}H NMR (acetonitrile-d_3): $\delta = 3.15$ ppm (d); ^{13}C-NMR (acetonitrile-d_3): $\delta = 44.7$, 122.6 ppm.

Loading of the 2-chlorotritylchloride-polystyrene with Fmoc-aminoalcohols and ethylenediamine. Optimal loading was obtained when working with a strict exclosure of water. A solution of Fmoc-Pheol (3 eq., 0.96 mmol) in dichloromethane was dried over Na_2SO_4 for 2 days. After filtration and evaporation, the residual Fmoc-Pheol was dried over P_2O_5 in high vacuum for 16 h, then dissolved in water-free DMF (1.2 ml) and added to 2-chlorotritylchloride-polystyrene-1% divinylbenzene (loading 1.6 mmol/g; 200 mg) in dichloromethane (1.2 ml). Pyridine (6 eq., 154 µl) was added and the reaction was stopped after 8 h by methanol addition for 30 min. After washing with DMF, MeOH, DMF, DCM, diethylether the loading was determined spectrophotometrically to be 0.25–0.30 mmol/g and the resin was used for the alamethicin syntheses. Prolongation of the reaction time from 8 to 48 h allowed a loading up to 0.88 mmol/g (Fmoc-alaninol). For the synthesis of peptide-(2-aminoethyl)amide intermediates for the *C*-terminal alamethicin-fullerene conjugates the resin was loaded with dry ethylenediamine (0.5 mol/l in dichloromethane) for only 5 min and free 2-chlorotritylchloride groups were capped by methanol.

Solid phase synthesis of alamethicin F30 and analogues. The use of Fmoc-amino acid fluorides for synthesizing Aib containing polypeptides is the method of choice [24] and a great progress compared with the classical segment condensations [15]. Here further improvements are presented and our general protocol for the automated solid phase synthesis of peptaibols via the generation of Fmoc-amino acid fluorides *in situ* using TFFH [16]. Examples for the *in situ* activation with TFFH for the synthesis of difficult peptide sequences without Aib residues have been reported in a short communication [16]. Because of the pronounced acid lability of Aib-Pro bonds [26,27] the 2-chlorotrityl anchor was used for all solid phase syntheses of peptaibols and side chain protected amino acids were Fmoc-Glu(tBu)-OH and Fmoc-Gln(Trt)-OH.

C-Terminal Alamethicin-f-30-fullerene-C_{60} and -C_{70} Conjugates

The syntheses of the two conjugates are outlined in Figure 1. The fully protected alamethicin-F30-2-aminoethylamide was synthesized on a PE Applied Biosystems Synthesizer 433A with a modified FastMoc 0.10 Ω MonPrev PK programme. The first residue Fmoc-L-phenylalanine (replacing Pheol) was coupled to resin loaded with 1,2-diaminoethane. All couplings were done with Fmoc-amino acid (10 eq.) and TFFH (10 eq.), both as solids in cartridges, diisopropylethylamine (20 eq.), in pure DMF for 60 min. Cleavage from the resin was done with hexafluoroisopropanol/dichloromethane (2 : 3) for 1 h and after partial evaporation the polypeptide was precipitated by *n*-hexane/diethylether (1 : 1). After lyophilization from tert-butylalcohol/water (4 : 1) and purification by RP-HPLC the side chain protected alamethicin-F30-2-aminoethylamide was acylated with 1,2-dihydro-1,2-methano-fullerene[60]-61-carboxylic acid-succinimide ester or 1,2-dihydro-1,2-methano-fullerene[70]-71-carboxylic acid-succinimide ester [21] in dichloromethane within 4 h. After precipitation with *n*-hexane, flash-chromatography on silica gel in chloroform/methanol (9 : 1) the protected conjugate (yield: 35%) was treated with trifluoroacetic acid/dichloromethane (1 : 1) containing 5% water and 2% triisopropylsilane.

Coordination ion spray mass spectra (CIS-MS) showed the expected molecular ions of *C*-terminal [Phe^{20}]-alamethicin F30-(2-aminoethylamide)-fullerene conjugates as ion adducts. Purified, fully protected alamethicin-fullerene C_{60} conjugate containing two Gln(Trt) and one Glu(tBu) residues showed Ag^+ adducts obtained by addition of aqueous $AgNO_3$ solution $[M + 3\,Ag]^{3+}$ 1214,5 and $[M + 2\,Ag]^{2+}$ 1768,0 amu; deprotected alamethicin-fullerene C_{60} conjugate, final product after addition of aqueous LiCl solution $[M + 3Li]^{3+}$ 933,5 and $[M + 2Li]^{2+}$ 1397,0 amu; deprotected alamethicin-fullerene-C_{70} conjugate, final product after addition of LiCl solution $[M + 3\,Li]^{3+}$ 973,5 and $[M + 2\,Li]^{2+}$ 1457,0 amu.

The covalent linkage between alamethicin F30-2-aminoethyl-amide and fullerene C_{60} carboxylic acid was proven by identification of the spin system of 1,2-diaminoethane by a clean TOCSY (t_M 70 ms) experiment: δ 8.63, 7.12 (2NH), δ 3.82, 3.75, 3.72, 3.64 (2 diastereotope CH_2,), δ 5.06 (fullerene CH-CO). In the NOESY (t_M 250 ms) experiment a NOE contact between

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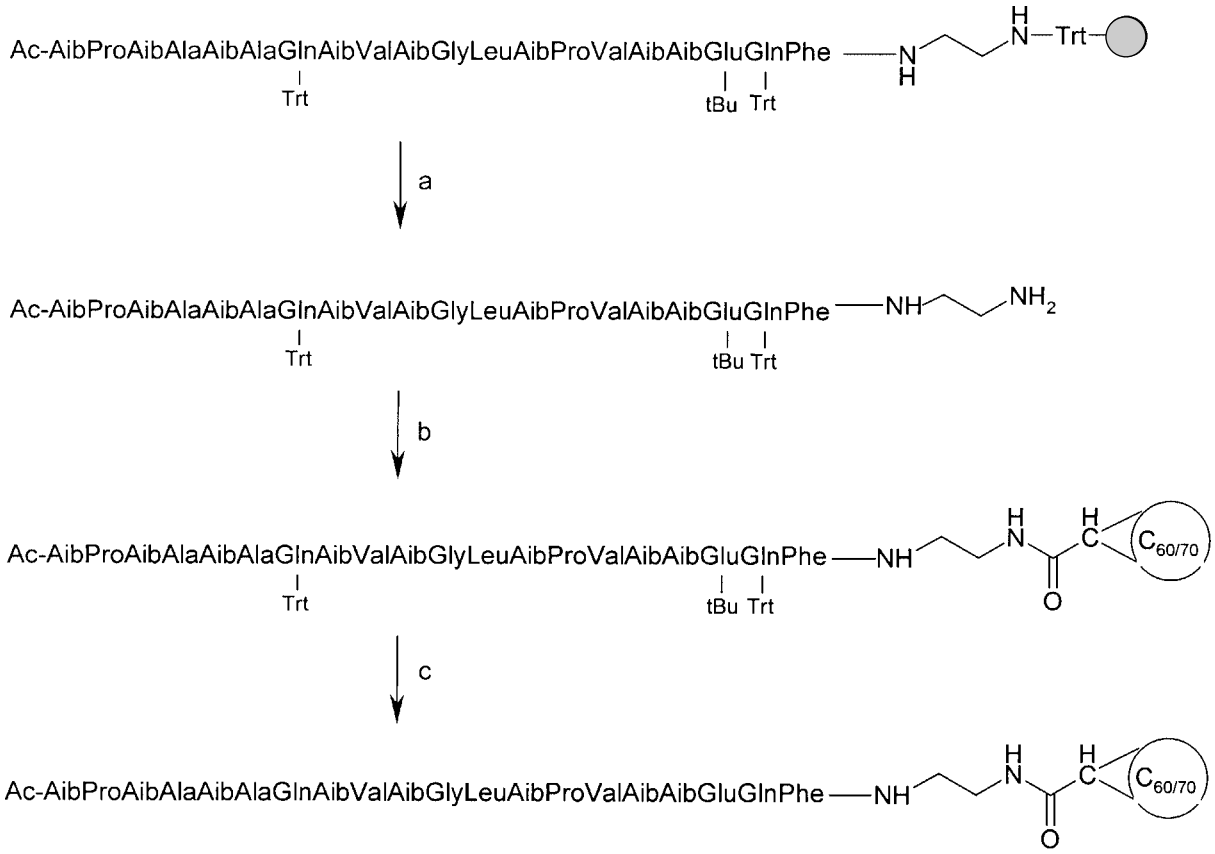

Figure 1 *C*-terminal active ester conjugation of fullerenes-C_{60} or C_{70} in solution to [Phe^{20}]-alamethicin F30-2-aminoethylamide synthesized on a 2-chlorotrityl-polystyrene resin using *in situ* TFFH activation: (a) cleavage with hexafluoroisopropanol/CH_2Cl_2, 1 h, (b) coupling of fullerene succinimide ester in CH_2Cl_2, 4 h; precipitation with *n*-hexane and flash chromatography on silica gel, (c) deprotection with TFA/CH_2Cl_2 (1 : 1) containing 5% H_2O, 2% triisopropylsilane.

NH (diamide) and CH-CO (fullerene) indicated the covalent linkage. The systematic names of the two *N*-acetylated 21-peptide amide conjugates are: alamethicin-F30-acid-β-[*N*-(1,2-dihydro-1,2-methanofullerene[60]-61-carbonyl]-aminoethyl] amide and alamethicin-F30-acid-β-[*N*-(1,2-dihydro-1,2-methanofullerene[70]-71-carbonyl)-aminoethyl amide.

N-terminal Alamethicin-F30-fullerene-C_{60} Conjugate

2-Chlorotritylchloride-resin was loaded with Fmoc-L-phenylalaninol and the alamethicin sequence was built up as outlined in Figure 2. However, instead of attaching acetyl-α-aminoisobutyric acid as the last residue, Fmoc-Aib-OH followed by Fmoc-6-aminohexanoic acid were coupled. The 21-peptide was deprotected and cleaved from the resin with trifluoroacetic acid/dichloromethane (1 : 1) containing 5% water and 2% triisopropylsilane. Precipitation with *n*-hexane/diethylether, lyophilization from tert-butyl alcohol/water (4 : 1) and purification by HPLC on a C18-reversed phase yielded the free 21-peptide.

N-terminal acylation was performed with fullerene-C_{60}-carboxylic acid (1 eq.) [21] which was dissolved in bromobenzene/DMF (2 : 1) and activated with HATU (1 eq.), diisopropylethylamine (10 eq.) for 30 min and then added to solid 22-peptide. After 15 h the crude conjugate was purified by flash-chromatography on silica gel using $CHCl_3$/MeOH (7 : 3) with 1% triethylamine as eluent. The product was characterized by CIS-MS [28].

C-terminal Alamethicin-lipopeptide Conjugates

C-terminal alamethicin-lipopeptide conjugates (Figure 3) were prepared from a highly purified alamethicin F50 component of a natural isolate [7]

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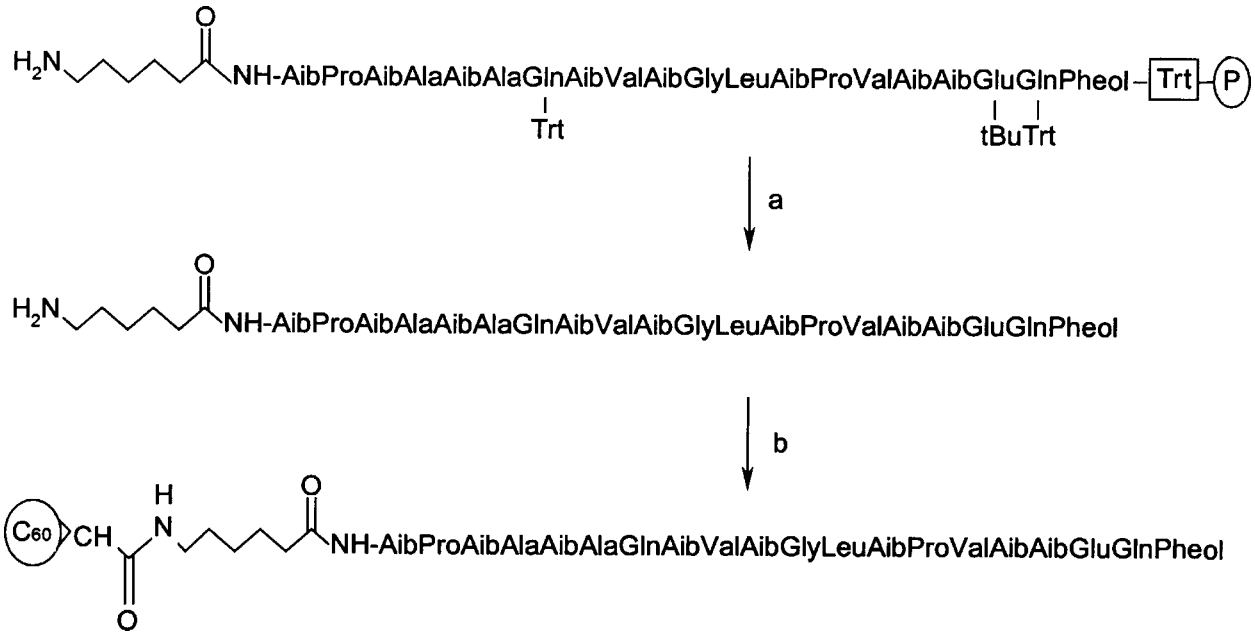

Figure 2 *N*-terminal conjugation of fullerene C60-carboxylic acid to [Aca^{21}]-alamethicin F30 synthesized on a 2-chlorotrityl resin: (a) cleavage and deprotection with TFA/CH_2Cl_2 (1 : 1) containing 5% H_2O, 2% triisopropylsilane, (b) after purification (RP-HPLC) conjugation in solution with fullerene C_{60}-carboxylic acid preactivated with HATU, DIEA, 15 h in bromobenzene/dimethylformamide.

Ac-AibProAibAlaAibAlaGlnAibValAibGlyLeuAibProValAibAibGlnGlnPheol-R

R = Pam3Cys-Aca-Gly-

Pam3Cys-Ser-Aca-Gly-

Pam3Cys = CH_2-O-CO-$(CH_2)_{14}$-CH_3
CH_2-O-CO-$(CH_2)_{14}$-CH_3
CH_2
S
CH_2
-OC-CH-NH-CO-$(CH_2)_{14}$-CH_3

Figure 3 *C*-terminal lipopeptide conjugates prepared by coupling tripalmitoyl-S-glyceryl-cysteinyl-peptides to purified natural alamethicin F50 isolate [7] using dicyclohexylcarbodiimide/4-dimethylaminopyridine and work-up by gel chromatography.

which showed only a minor microheterogeneity in RP-HPLC and ESI-MS analysis. Alamethicin F50 has a glutamine residue in position 18, where alamethicin F30 has a glutamate, therefore alamethicin F50 has only one *C*-terminal hydroxy group for esterification with lipopeptides. The lipopeptides Pam_3Cys-Aca-Gly and Pam_3Cys-Ser-Aca-Gly were prepared according to published protocols

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[20,29,30] and analysed by ESI-MS [31]. The diastereomer with (R)-configuration at C2 of the 2,3-dihydroxypropyl-moiety in Pam_3Cys-Ser has been shown to exhibit higher adjuvant activity than the corresponding (S)-configurated analogue [32]. Here, the mixture of diastereomers of Pam_3Cys was used, which is not expected to influence the BLM experiments.

Pam_3Cys-Ser-Aca-Gly-OH (5.84 mg, 5 μmol) [33] was esterified with natural alamethicin F50 (9.82 mg; 5 μmol) from *Trichoderma viride* NRRL 3199 [7] by activation with *N*,*N*′-dicyclohexylcarbodiimide (5 μmol) and 4-dimethylaminopyridine (5 μmol) in dimethylformamide. After 15 h the reaction mixture was chromatographed on Sephadex LH 20 using dichloromethane/dimethylformamide (1 : 1) and fractions were detected by thin-layer chromatography (spraying with water and TDM reagent). The conjugate was free of alamethicin and low molecular mass impurities. The molecular mass of $m/z = 3124$ $[M + H]^+$ was confirmed by ESI-MS. The final yield was low (1.13 mg).

Using the same procedure Pam_3Cys-Aca-Gly-OH (5.4 mg; 5 μmol) [20] was esterified with alamethicin F50 and the conjugate isolated in low yield (0.83 mg). The amino acid analysis of the hydrolysate of alamethicin F50-(Pam_3Cys-Aca-Gly) was compared with that of natural alamethicin F50 and showed the expected increase in the Ala/Gly ratio from 1.00/0.54 to 1.00/0.97 ESI-MS indicated $m/z = 3027$ $[M + H]^+$.

Analytical Methods

Analytical HPLC and semipreparative separations were done on a system gold instrument of Beckman with autosampler and diode array detector. Analytical column: 250 × 2 mm, nucleosil 300 C_{18} (5 μm), 200 μl/min, linear gradient water (0.1% TFA) (A) /acetonitrile (0.08% TFA) (B) from 10% to 100% B within 45 min. Semipreparative column: 250 × 8 mm, nucleosil 300 C_{18} (5 μm) from Grom (Herrenberg, Germany), 4 ml/min, same eluent system.

Electrospray mass spectra were taken on a API III TAGA 6000E triple quadrupole mass spectrometer with an ion spray ionization source (Sciex, Thornhill, Ontario, Canada). Samples were dissolved in acetonitrile/water (1 : 1) or methanol/1% formic acid or tert-butylalcohol/water (4 : 1) (0.1 mg/ml) and injected via a capillary and a medical infusion pump (Harvard Apparatus) at a flow rate of 5 μl/min. Whereas alamethicin-lipopeptide conjugates are easily ionized [31], ionization of the fullerene-alamethicin conjugates had to be enhanced by metal complexation (CIS-MS) [28]. ^1H- and ^{13}C-NMR spectra (TOCSY, NOESY) were measured on a Bruker AMX spectrometer (400 MHz). Amino acid analyses were performed on a fully automated amino acid analyser 420 A (Applied Biosystems). Samples: 400–1000 pmol in 30 μl, hydrolysis with 6N HCl in gas phase at 160 °C for 90 min. Detection after on-line derivatization with phenylisocyanate and microbore HPLC.

Measurements of the Voltage-dependent Ion Conductance in Black Lipid Bilayers

Lipids and standard solutions. 1-Palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (1,2-POPC), 1,2-palmitoyl-sn-glycero-3-phosphocholine (1,2-DPPC) and 1,2-dioleyl-sn-glycero-3-phosphatidylethanolamine (DOPE) were from Avanti Polar Lipids, Birmingham, USA, and used without further purification. Standard solutions in *n*-hexane puriss. were stored at −40 °C. Aliquots were taken and *n*-hexane removed by a N_2-stream and the residue taken up in *n*-decane puriss. Lipid solutions had a final concentration of 12 mg/ml and for each BLM preparation 5 μl of this solution was used. For 1,2-POPC/1,2-DPPC mixtures, 5, 10, 15 and 20 mol % 1,2-DPPC was used. In the case of slight turbidity one drop of ethanol p.a. improved the solubility of 1,2-POPC in *n*-decane without damaging the BLM. KCl, $CaCl_2$, ethanol, *n*-hexane, *n*-decane were from Riedel-de Haen, Seelze, Germany, and HEPES and Tris from Sigma, St Louis, USA.

Alamethicin (standard used for comparison) was dissolved in ethanol/water (1 : 1) at concentrations of 2×10^{-5} g/ml or 10^{-4} g/ml. The fullerene conjugates of alamethicin were dissolved in water-free methanol and the lipopeptide conjugates were dissolved in water-free ethanol (10^{-4} g/ml). For the BLM experiments samples of 5 and 10 μl were applied (final concentration 2×10^{-9} M for channel experiments).

BLM experiments and evaluation of the single channel measurements. In the first experiments virtually solvent-free planar lipid bilayers were formed on a hole of 0.2 mm^2 in a teflon sandwich septum [4,17]. Actual experiments with lipopeptide and fullerene conjugates were carried out in polymethylmethacrylate (PMMA, Perspex^R) cuvettes with polished holes of 0.1 mm diameter in the wall separating the *cis* from the *trans* site. Before

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adding the lipids from both sites the PMMA material was treated with silicon spray to cover its hydrophilic surface and thus to allow experiments for several hours. After adding the lipids the solvent was evaporated and the electrolyte filled in. The temperature was 22 °C, if not indicated otherwise. For temperature dependent measurements the cuvette was placed in a metal block connected with a thermostat. The whole apparatus was covered with a Faraday cage to minimize electric disturbances.

At the beginning of each experiment a BLM was prepared by adding a droplet of lipid on the surface of the electrolyte solution of the *cis* compartment followed by up-and-down movement of the cuvette. The addition of the solutions of the fullerene conjugated channel formers was done to the *cis*-compartments with respect to the grounded *trans*-compartment, whereas the lipopeptide conjugates of alamethicin were added to the *trans* site. Ag/AgCl electrodes linking the electrolyte solutions (1 M NaCl and 10 mM Tris/HCl, pH 7.0) were daily treated with fresh 0.1 M HCl.

The data collected from voltage-dependent channel experiments were evaluated with self-written program packages with respect to open probabilities and mean lifetimes of the blocked states as described [1,7].

RESULTS AND DISCUSSION

Design of Alamethicin Conjugates with Fullerenes and Lipopeptides

Various templates decorated with α-helices including alamethicins have been reported to exhibit ion conducting properties in membranes or vesicles [34,35]. In contrast to these constructs our aim was to achieve pore state stabilizations without attachment of alamethicin monomers to templates or to use otherwise covalently linked oligomers. Our idea was to design *N*- or *C*-terminally modified alamethicin monomers that are still able to self-assemble and to form the characteristic voltage-dependent pores in lipid bilayers. A conjugation of alamethicin with neutral and large lipophilic molecules with known properties was more promising than the introduction of multiple charges or highly polar groups with uncertain structural and electrical effects.

It was also planned to elongate alamethicin molecules with peptide epitopes, for example with the partially α-helical *C*-terminus of the foot-and-mouth disease VP1 protein [35], and to immobilize such constructs by adding corresponding antibodies to the *cis*- or *trans*- compartments in bilayer experiments. This idea remains in our working programme because very positive results had been observed in experiments with biotinylated alamethicins immobilized by streptavidin complexation. However, interactions between specific small ligands of such alamethicin conjugates and very large protein receptors raise a number of problems both in experiments and in the interpretation of results although a strong modulation of the kinetics of pore formation can be expected. Finally, our molecules of choice for the conjugation with alamethicins were functionalized fullerenes C_{60} and C_{70} [21] and the synthetic *N*-terminal head group of bacterial lipoprotein carrying three fatty acids [20,29,33].

C-terminal Alamethicin-fullerene Conjugates, Novel Constructs for Synthetic Ion Channels

The diameter of the hydrophobic C_{60} ball of 10 Å is comparable to the diameter of an α-helix as shown by a space-filling model of the alamethicin F30-fullerene C_{60} conjugate. Therefore one could expect a stabilization according to the barrel stave-model and flip-flop gating model, which assumes ion conducting aggregates consisting of α-helices arranged in parallel [1,11].

The synthesis of such conjugates was first performed via esterification of natural alamethicin F50 (microheterogeneous isolate with *C*-terminal phenylalaninol as the only functional group) [7] with glycine followed by coupling with fullerene C_{60}-N-hydroxysuccinimide ester (H.G. Ihlenfeldt, unpublished results). Since the natural alamethicin F50 isolates possess only one reactive phenylalaninol hydroxy group, a site specific *C*-terminal linkage was possible, but the yield was moderate and the product microheterogeneous. Therefore, general protocols were developed for total syntheses of alamethicins which are linked *C*- or *N*-terminally with either fullerene-C_{60} or C_{70}-carboxylic acids [21] via short spacer molecules (Figures 1 and 2). Synthesis of *C*-terminal alamethicin-fullerenes was planned in such a way that the natural *N*-terminus with the *N*-acetyl-Aib-Pro cap was preserved and for the *C*-terminus L-phenylalanine instead of L-phenylalaninol was introduced and the short spacer ethylenediamine (Figure 1). *C*-terminal alamethicin F30-fullerene C_{60} and C_{70}-conjugates were obtained

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via coupling of fullerene carboxylic acid succinimide esters to purified side chain protected [^{20}Phe]-alamethicin F30-2-aminoethyl amide.

Alamethicin F30 was built up on a 2-chlorotrityl-chloride-polystyrene resin loaded with 1,2-diaminoethane (Figure 1). Fmoc-amino acids were coupled via our *in situ* TFFH activation protocol [16] and after very mild cleavage by hexafluoroisopropanol/dichloromethane (1 : 1) RP-HPLC yielded an intermediate with excellent purity for coupling with the two fullerene active esters. After the conjugation step the alamethicin-fullerene conjugates were deprotected by a short treatment with trifluoroacetic acid/dichloromethane (1 : 1) containing 5% water and 2% triisopropylsilane.

The characterization by ESI-MS had to be supported by metal ion additives because of the low ionizability of fullerene derivatives. This special ionization procedure for lipophilic compounds is known as coordination ion spray (CIS) [28]. To summarize, intense doubly and triply charged molecular ion peaks were obtained for the Ag^+ salts (addition of aqueous $AgNO_3$ solution) of the fully protected alamethicin-fullerene conjugates. The deprotected alamethicin F30-fullerene C_{60} and C_{70} conjugates were ionized by addition of LiCl solution to give the expected and intense doubly and triply charged molecular ion peaks.

Synthesis of *N*-terminal Conjugates of Alamethicin with Fullerene

For the synthesis of *N*-terminally modified alamethicin-F30 the natural *C*-terminus was preserved. Therefore, 2-chlorotritylchloride-polystyrene was loaded with Fmoc-L-phenylalaninol and the complete alamethicin sequence was built up again with Fmoc-amino acids and *in situ* activation with TFFH [16]. After Fmoc-^{1}Aib-OH, Fmoc-ε-aminocaproic acid was introduced to provide a relatively short lipophilic spacer. After cleavage and RP-HPLC purification of the prolonged alamethicin, fullerene C_{60}-carboxylic acid was coupled as the HATU active ester in solution and the conjugate was purified by flash chromatography on silica gel and analysed. The preformed fullerene-HATU active ester reacted only with the *N*-terminal amino group and not with the *C*-terminal hydroxy group of phenylalaninol.

Synthesis of Alamethicin-lipopeptide Conjugates, Mimetics of Bacterial Cell Wall Components with Ion Channel Activity

The lipoprotein of Gram-positive bacteria is a 55 residue membrane protein which connects the murein layer with the outer lipid membrane. The part of the lipoprotein molecule responsible for adjuvant activity resides in the short three fatty acids containing *N*-terminal lipopeptide S-(2,3-bis(palmitoyloxy)-(2*R* and *S*)–propyl)-N-palmitoyl-(*R*)-Cys-(*S*)-Ser-Ser-Asn-Ala which has been prepared by chemical synthesis including a large series of analogues [20,29]. All synthetic lipopeptides carrying the head group tripalmitoyl-S-glyceryl-cysteine (Pam_3Cys) such as the water soluble Pam_3Cys-Ser-$(Lys)_4$ [36] are non toxic, stable and potent immunostimulators, which activate membrane bound Toll-like receptor 2 (TLR2) of the innate immune system [37]. Highly efficient modular lipopeptide vaccines have been constructed by coupling the adjuvant Pam_3Cys to B-, T-helper- and CTL-epitopes [38–41]. Biophysical studies of lipopeptides in artificial and biological membranes revealed self-assembling and membrane anchoring properties: isotherms of mixed monolayers with Pam_3Cys and lipids were analysed with the film balance [42]. Confocal fluorescence microscopy with FITC-labelled lipopeptides showed patching and capping on lymphocytes [43] and fluorescence quenching experiments were carried out in vesicles [44]. EELS spectroscopy showed a fast uptake of a fluorinated lipopeptide in outer and inner membranes of B-lymphocytes within 2 min and detectability up to 24 h [45]. Furthermore, Pam_3Cys derivatives proved to be excellent membrane anchors to covalently link sensor substrates and lipid bilayers [46,47].

These favourable membranophilic properties prompted us to synthesize alamethicin-lipopeptides conjugates in solution (Figure 3). The lipoamino acid Pam_3Cys was elongated *C*-terminally by short spacer peptides Aca-Gly and Ser-Aca-Gly and the resulting lipotri- and lipotetrapeptides were esterified with the hydroxy group of the *C*-terminal phenylalaninol of natural alamethicin F50. The two analytically characterized conjugates have molecular masses of 3026 and 3123 Daltons and they are soluble in dichloromethane and dimethylformamide.

Voltage-dependent Channel Formation of Alamethicin and Derivatives

Synthetic ion channels contribute to the understanding of structure and function of natural transmembrane ion channels. Furthermore, robust synthetic ion translocating components are desirable for artificial membranes, biosensors and nanostructures. Microbial channel formers such as gramicidin

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A and some peptaibols and also transmembrane segments of channel proteins may be used to mimic at least in part the properties of the more complex and ligand gated biological ion channels. Among the various synthetic approaches and models we should like to mention the Ser/Leu containing α-helical peptides of De Grado [48] and Mutter's template-assembled synthetic protein (TASP) concept applied by Montal [49]. *C*-terminal modification of alamethicin with metal complexing moieties [50] lead to channels with a prolonged lifetime and higher selectivity for Ca^{2+} in the case of alamethicin-pyromellitate [51]. Longer lifetimes resulted from two covalently linked alamethicins [52] and redox-sensitive alamethicin channels have been reported to result from esterification of alamethicin with ferrocencarboxylic acid [53].

Obviously, alamethicin continues to be one of the most interesting tools for biophysics and sensorics related to potential-dependent, transmembrane ion conducting systems. Our examples of *C*-terminal alamethicin-fullerene and lipopeptide conjugates are template-free constructs allowing a stabilization of self-aggregating ion channels with preferred conductance states in black lipid membranes which can be observed for longer time periods than those of natural alamethicin and related peptaibols.

Single channel Experiments with C-terminal Alamethicin-fullerene Conjugates. Both of the *C*-terminal alamethicin F30-fullerene C_{60} and C_{70} conjugates exhibit channel activities which show pronounced differences in comparison with the well characterized channel properties of natural or synthetic alamethicin [1]. Multi-level bursts of channel opening occur at higher voltage (150 mV) in POPE/POPC membranes in symmetric 1 M KCl. The most important fact is that the derivatization with fullerenes results in the stabilization of defined oligomer assemblies. In voltage/current experiments the fullerene-C_{60} conjugate shows very frequently consecutive transitions between two defined and neighboured lower conductance states (Figure 4a) when compared with natural alamethicin, whereas the fullerene-C_{70} conjugate shows channel states with remarkable long lifetimes in the region of seconds (Figure 4b). It is of particular interest that the onset of current fluctuations

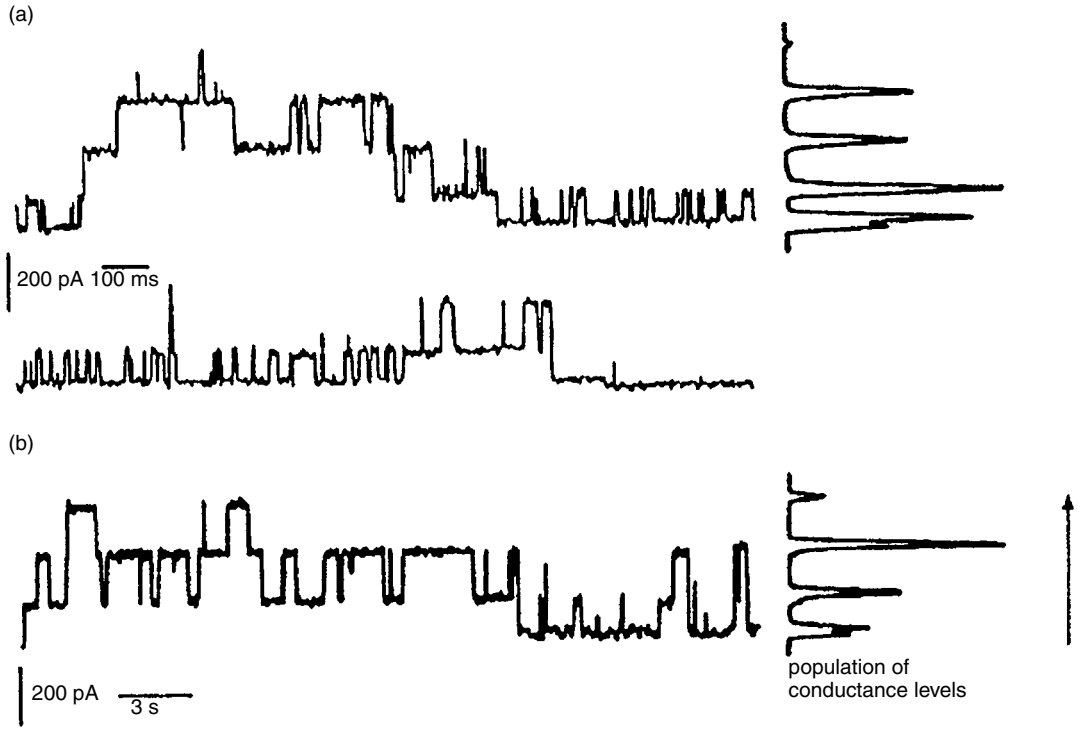

Figure 4 Single channel current fluctuations of *C*-terminal alamethicin F30-fullerene C_{60} (a) and fullerene C_{70} (b)-conjugates. Experimental conditions: BLM of 1-palmitoyl-2-dioleyl-glycero-3-phosphatidylcholine/1,2 dioleyl-glycero-3-phosphatidylethanolamine (9 : 1, mol %) dissolved in decane; electrolyte: 1 M KCl, 10 mM HEPES, pH 7.2; temperature 18 °C; concentration of conjugate $c = 2 \times 10^{-9}$ M.

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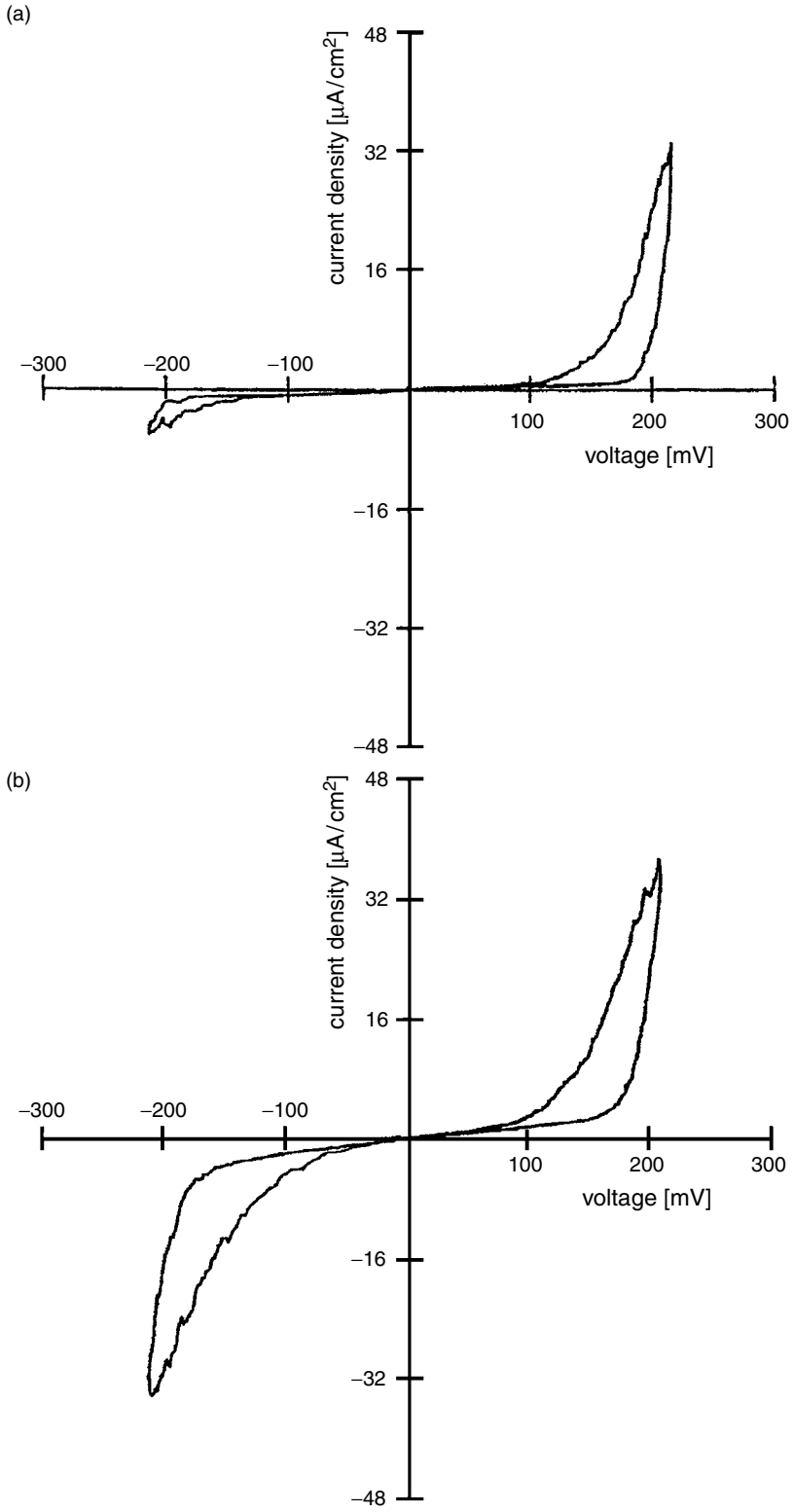

Figure 5 Current-voltage (CV) curves after addition of the *C*-terminal alamethicin F30-fullerene C_{60} conjugate to the *cis*-site of the BLM (a) and after 30 min (b) at negative potential indicating transmembrane translocation. Conditions: BLM as in Figure 5; 200 mM KCl, temperature 23°–24 °C; concentration of conjugate $c = 2 \times 10^{-9}$ M.

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is observed at nanomolar conjugate concentrations which are lower by more than a factor of 10 when compared with nonmodified alamethicin. Obviously, the alamethicin-fullerene conjugates have a higher affinity to the lipid membrane phase than natural alamethicin. On the other hand the current fluctuations have a higher induction voltage due to the big fullerene balls and stronger asymmetry in current voltage curves is observed immediately after addition to one compartment (Figure 5).

Single channel experiments with* N*-terminal fullerene alamethicin conjugate. The *N*-terminal fullerene-C_{60} alamethicin F30 conjugate shows channel forming properties which are totally different from those of the *C*-terminally modified alamethicins. During single channel experiments conductance jumps are observed at high voltage. Again the conjugates are incorporated into the membrane, but no stable ion channel states can be observed. In this context we refer to a recent publication describing fullerene-lipid — biotin conjugates which fully incorporated into membranes [54]. Since the typical fluctuation pattern of alamethicin disappeared one must conclude that *N*-terminal modification with fullerene leads to instability of the channel formation possibly by incorporation of the ball part in different depths within the bilayer membrane.

Single channel experiments with* C*-terminal alamethicin-lipopeptide conjugates. The lipopeptide conjugate of alamethicin F50 (Figure 3) is a large molecule which is not expected to diffuse across the membrane or to exert flip-flop motions within the membrane. One would expect ion channel formation only at negative transmembrane voltage after addition of the conjugate to the *trans* site of the lipid bilayer, because alamethicin is coupled to tripalmitoyl-S-glyceryl-cysteine via phenylalanine on the positive pole of the helix dipole [9–11]. The BLM experiment in 1,2-POPC membranes confirmed exactly this expectation. Firstly, there are no bursts typical for alamethicin and only a small number of events (Figure 6). Secondly, compared with alamethicin the lipopeptide conjugate shows a strong shift of the pore states distribution to lower conductance states with $\upsilon = 1–4$. The longest open channel times were observed for $\upsilon = 1$ and $\upsilon = 2$.

Additional differences were observed in measurements using lipid mixtures. Alamethicin is more likely surrounded by the more fluidic component 1,2-POPC, whereas the lipopeptide conjugate carries covalently the less fluidic component Pam_3Cys. As a consequence in pure 1,2-POPC the lipopeptide conjugate exhibited frequently very long lasting basic conductance states and short events, whereas alamethicin showed its typical bursts.

For the experiments in $CaCl_2$ as electrolyte $\nu = 2$ was the lowest conductance state, because $\nu = 1$ is not permeable for Ca^{2+} and Cl^-. Open times and conductance states and the missing bursts are similar to the BLM experiments performed in KCl.

Without going into details, we report that we also synthesized *N*-terminally linked lipopeptide conjugates of the bee venom component melittin. Former experiments with α-helical polypeptide

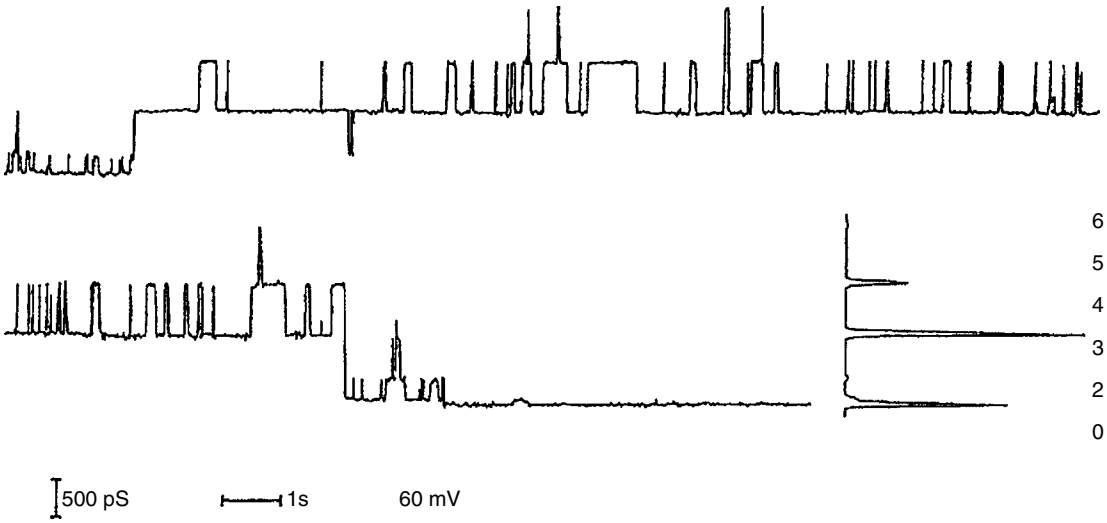

Figure 6 Single channel current fluctuations of *C*-terminal alamethicin-lipopeptides conjugates in solvent free POPC bilayer membranes.

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melittin showed that resolved short and burst like single channel events could be recorded under special conditions [55]. In the conditions used here for recording channel formation by alamethicin and its conjugates melittin showed no resolved pore states. However, the lipopeptide conjugate Pam_3Cys-Ser-Aca-Gly-melittin 1–26 allowed the recording of two stabilized states similar to those observed for the lipopeptide conjugate of alamethicin but with lower conductance and much shorter open times.

In summary the experiments with our lipopeptide conjugates confirm the channel model of Baumann and Müller [56] and they are in accordance with the particular membrane properties of the lipid anchor Pam_3Cys derived from bacterial lipoprotein.

CONCLUSION

From our experimental results one can summarize that we successfully designed, synthesized and characterized novel examples of functional fullerene-biopolymer conjugates. In the case of alamethicin $C_{60/70}$-fullerene conjugates the *C*-terminal attachment of the lipophilic balls results in a pronounced channel stabilization as expected, whereas the *N*-terminal modification results in a drastic disturbance of the single channel current fluctuations.

Our examples give proof of the concept for a generally applicable stabilization strategy for ion channels formed by α-helical and transmembrane spanning polypeptides. This conclusion is supported by an additional observation. In an extended study we synthesized also a *C*-terminally fullerene C_{60} modified trichotoxin derivative. It should be emphasized that the natural peptaibol mixture of trichotoxin and the uniform synthetic trichotoxin A40 [57] do not exhibit resolved conductance states such as those of alamethicin. Indeed, the first biophysical experiments indicated that trichotoxin-fullerene C_{60} conjugates form stabilized ion conducting channels.

The preference of the *C*-terminus and not the *N*-terminus with respect to the introduction of pore state stabilizing structural elements is not unexpected due to some additional former observations. For example a *C*-terminal fixation of monomers within the pore aggregates was observed also for carboxyproxid derivatives with direct linkages to phenylalaninol of alamethicin [19], whereas *C*-terminally more mobile derivatives with a spacer between carboxyproxid and the hydroxy group of phenylalaninol had a strongly destabilizing influence. Peptaibols lacking *C*-terminal aromatic amino alcohols also show drastically reduced lifetimes of pore states compared with alamethicin, examples are trichotoxins, hypelcins and trichobrachins. On the other hand, the introduction of larger aromatic groups such as tryptophanol instead of phenylalaninol may stabilize the pores as shown for the trichorzianines [58]. *C*-terminal conjugation of alamethicin has a particularly strong influence on channel stabilization.

Our experimental results of novel template-free voltage-dependent ion conducting systems may be of some value for the future design of artificial ion channels based on self-assembling α-helices. Furthermore the conjugates may be of interest for investigating the biological effects of peptaibols on cells, such as the activation of membrane bound enzymes, which was observed for alamethicin, suzukacillin and trichotoxin which activate ciliary guanylate cyclase from *Paramecium* [59].

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