Self-assembled, cation-selective ion channels from an oligo(ethylene glycol) derivative of benzothiazole aniline

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Abstract

This paper describes the spontaneous formation of well-defined pores in planar lipid bilayers from the self-assembly of a small synthetic molecule that contains a benzothiazole aniline (BTA) group attached to a tetra-ethylene glycol (EG4) moiety. Macroscopic and single-channel current recordings suggest that these pores are formed by the assembly of four BTA-EG4 monomers with an open pore diameter that appears similar to the one of gramicidin pores (~0.4 nm). The single-channel conductance of these pores is modulated by the pH of the electrolyte and has a minimum at pH ~3. Self-assembled pores from BTA-EG4 are selective for monovalent cations and have long open channel lifetimes on the order of seconds. BTA-EG4 monomers in these pores appear to be arranged symmetrically across both leaflets of the bilayer, and spectroscopy studies suggest that the fluorescent BTA group is localized inside the lipid bilayers. In terms of biological activity, BTA-EG4 molecules inhibited growth of gram-positive Bacillus subtilis bacteria (IC50 ~50 μM) and human neuroblastoma SH-SY5Y cells (IC50 ~60 μM), while they were not toxic to gram-negative Escherichia coli bacteria at a concentration up to 500 μM. Based on these properties, this drug-like, synthetic, pore-forming molecule with a molecular weight below 500 g mol−1 might be appealing as a starting material for development of antibiotics or membrane-permeating moieties for drug delivery. From a biophysical point of view, long-lived, well-defined ion-selective pores from BTA-EG4 molecules offer an example of a self-assembled synthetic supramolecule with biological function.

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1. Introduction

Ion channel proteins mediate transport of ions across cell membranes and play a critical role in physiological processes such as signal transduction, regulation of membrane potentials, and cell proliferation [1,2]. Due to the importance of ion channels in applied fields, such as biosensing, and for the pathogenesis of certain diseases [3–15], semi-synthetic as well as synthetic versions of pore-forming molecules or artificial ion channels are attracting increasing attention [16–24]. Additional applications of synthetic ion channels or engineered biological pores include their use as antimicrobial agents, drug delivery agents, fluorescent dyes, and components of bio-inspired batteries [13,14,25,26].

With regard to the synthesis of ion channel-forming molecules, two approaches have been applied: 1) covalent attachment of pore-forming residues to a molecular scaffold or template, which can have additional functionality as a selectivity filter [24,27–31], or 2) self-assembly of small molecules to pores, which can conduct ions across lipid membranes [27,30,32–34].

With regard to the templated approach, several research groups developed synthetic ion channels and pores by attaching amphiphilic moieties to cyclodextrin [29,35–37], crown ethers [38], calixarenes [39], or cucurbituril templates [40]. Some of these templated channels contained oligo(ethylene glycol) (OEG) groups or moieties with alternating oxygen and carbon molecules, which were typically attached to a cyclodextrin scaffold. Lehn et al. proposed in 1993 that OEG groups grafted on a cyclodextrin scaffold could form ion channels [41]. Gin’s group created ion channels composed of a β-cyclodextrin template with oligoether chains attached to the "primary face of cyclodextrin" through amine linkages [29]. Liposome flux experiments revealed that this molecule was able to induce transport of Na+ ions across lipid membranes. Recently, Badi et al. demonstrated by single-channel recordings through planar lipid bilayers that per-2,3-heptyl-β-cyclodextrin scaffolds modified with poly(ethylene oxide) (PEO) chains, could form ion channels in lipid membranes [42].
These authors also showed that PEO moieties by themselves did not form pores at the equivalent concentrations, demonstrating the importance of the cyclodextrin scaffold.

With regard to self-assembly of synthetic ion-channel-forming molecules, Neumann and colleagues have shown by fluorescence spectroscopy and ion channel recordings through planar lipid bilayers that molecules with an ester bond between a fatty acid and an OEG moiety can form ion channels [43]. They also demonstrated by agar well diffusion assays that these molecules had antibacterial activity against gram-positive bacteria [44]. In addition, Yang et al., Hirata et al., and Kim et al. have demonstrated by X-ray diffraction, TEM, molecular dynamics (MD) simulations, and liposome-based fluorescence assays that molecules with alternating oxygen and carbon atoms, including OEG, could interact with lipid membranes and form ion channels [45–47].

Here, we introduce a small synthetic molecule that self-assembles and forms ion-selective channels with surprisingly well-defined single-channel conductance in phospholipid membranes. This molecule has a molecular weight below 500 g mol \(^{-1}\) and is based on a tetra-ethylene glycol moiety that is attached to a relatively hydrophobic benzothiazole aniline (BTA) group (Fig. 1A). We chose this OEG derivative of BTA, which we call BTA-EG\(_{4}\), based on our ongoing research in the context of Alzheimer’s disease and HIV transmission, which revealed that BTA-EG\(_{4}\) can readily cross cellular membranes and affect disease-related biological processes after uptake into mammalian cells [48–50]. Based on the observed membrane permeability, we focused here on the effect of BTA-EG\(_{4}\) on planar lipid bilayer membranes under well-defined experimental conditions. We show by single-channel recordings that BTA-EG\(_{4}\) forms well-defined ion pores that are selective for monovalent cations. We characterize the open lifetime, the approximate number of monomers in each pore, the conformational stability of the pores, and the pH-dependence of the single-channel conductance. Finally, we show that this self-assembling, pore-forming molecule is toxic to the gram-positive bacterium *Bacillus subtilis* and to a human neuroblastoma cell line (SH-SY5Y), while it had no detectable toxic effect on the gram-negative bacterium *Escherichia coli*. The cytotoxic concentration of BTA-EG\(_{4}\) was similar to the concentrations necessary to observe ion channel activity, suggesting pore formation as the mechanism of toxicity.

### 2. Materials and methods

#### 2.1. Synthesis of BTA-EG\(_{4}\) molecule

**2.1.1. 2-((2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethyl toluenesulfonate**

In a clean, dry 1 L round bottom flask equipped with a stir bar, we dissolved tetra-ethylene glycol (10.0 g, 51.5 mmol) in 500 mL dry dichloromethane (DCM) and stirred at room temperature. After 5 min, potassium iodide (1.71 g, 10.3 mmol), Ag\(_{2}\)O (17.9 g, 77.2 mmol), and p-toluenesulfonyl chloride (10.8 g, 56.6 mmol) were successively added to the reaction flask. The reaction mixture was stirred vigorously for 2 h, filtered through celite to remove the solids and concentrated in vacuo. The residue was purified via silica gel chromatography (100% DCM to 95:5 DCM:CH\(_{3}\)OH) giving 2-((2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl toluenesulfonate as a colorless oil (13.2 g, 74%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta = 7.74\) (d, 8.0 Hz, 2H), 7.30 (d, 8.0 Hz, 2H), 4.11 (t, 4.8 Hz, 2H), 3.66–3.53 (m, 12H), 2.79 (s, 1H), 2.39 (s, 3H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta = 145.04, 133.17, 130.10\) (2C), 128.19 (2C), 70.95, 70.79, 70.70, 69.49, 68.88, 21.87. ESI-MS (m/z) calculated for C\(_{34}\)H\(_{42}\)O\(_{7}\)S: [M]\(^+\) 380, 348, [M + H\(^\pm\)] 389.66, [M + Na\(^\pm\)] 363.94, 357.08.

**2.1.2. 2-((2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethyl toluenesulfonate**

We combined 2-((2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl toluenesulfonate (12.01 g, 34.5 mmol), sodium iodide (20.7 g, 137.9 mmol) and 200 mL dry acetone in a clean, dry round bottom flask and heated to reflux with vigorous stirring. After 12 h the reaction was cooled to room temperature and diluted with 100 mL ethyl acetate. The organic phase was washed with 10% Na\(_2\)S\(_2\)O\(_3\) solution and dried in anhydrous Na\(_2\)SO\(_4\), filtered through celite to remove the solids, and concentrated in vacuo giving 2-((2-(2-hydroxyethoxy)ethoxy)ethoxy)ethanol as a pale yellow oil (5.61 g, 54%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta = 3.73-3.58\) (m, 14H), 3.24 (t, 2H), 2.59 (s, 1H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta = 72.70, 72.19, 70.90, 70.76, 70.58, 70.39, 61.94, 3.07.

**2.1.3. BTA-EG\(_{4}\)**

A microwave reaction tube was charged with 2-((2-(2-iodoethoxy)ethoxy)ethoxy)ethanol (1.47 g, 4.83 mmol), benzothiazole aniline (3.49 g, 14.5 mmol), potassium carbonate (3.34 g, 24.2 mmol) and 20 mL dry THF. The tube was then equipped with a small stir bar, sealed and placed in a microwave reactor. The reaction was heated and kept at 125 °C for 2 h. The reaction was cooled to room temperature and filtered to remove the solids. The solids were washed several times with DCM until the filtrate was colorless. The combined organic layers were concentrated in vacuo and purified by column chromatography to give the desired BTA-EG\(_{4}\) compound as a yellow solid (1.13 g, 56%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta = 7.87\) (d, 8.8 Hz, 2H), 7.83 (d, 8.4 Hz, 1H), 7.63 (s, 1H), 7.23 (d, 8.4 Hz, 2H), 6.68 (d, 8.8 Hz, 2H), 3.76–3.64 (m, 14H), 3.37 (t, 5.2 Hz, 2H), 2.47 (s, 3H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta = 168.03, 152.64, 150.92, 134.87, 134.47, 129.13\) (2C), 127.70, 122.88, 122.03, 121.41, 112.82 (2C), 72.86, 70.88, 70.69, 70.43 (2C), 69.64, 61.91.
43.32, 21.70. HR-ESI-MS \((m/z)\) calculated for \(\text{C}_{22}\text{H}_{28}\text{N}_{2}\text{O}_{4}\text{SNa} \ [\text{M} + \text{Na}]^+\) 439.1662; found \([\text{M} + \text{Na}]^+\) 439.1660.

2.2. Materials

We purchased cesium chloride (CsCl) from International Biotechnologies Inc.; sodium chloride (NaCl), and potassium bromide (KBr) from Fluka; and potassium chloride (KCl), HEPES, and hexane from Fisher Scientific. All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

2.3. Formation of planar lipid bilayers

We used 1,2-diphtanyloyl-sn-glycero-3-phosphatidylcholine (DiPhyPC) from Avanti Polar Lipids (Alabaster, Alabama) for the formation of all bilayers in this work. We prepared planar lipid bilayers by the “painting method” by applying a solution of 20 mg mL\(^{-1}\) DiPhyPC in n-heptane or n-decane over an aperture with a diameter of ~250 \(\mu\text{m}\) in a Delrin cup (Warner Instruments) [11,51]. Before bilayer formation, we pretreated these apertures with a droplet of a solution of 20 mg mL\(^{-1}\) DiPhyPC in n-hexane. Both compartments of the chamber (cis and trans) were filled symmetrically with electrolytes containing 1.0 M concentrations of various salts with 10 mM HEPES buffer adjusted to pH 7.4 with HCl unless indicated otherwise. For each experiment, we confirmed the stability of the lipid membrane by applying a voltage of \(\pm 100\text{ mV}\) for 10 min before addition of reagents to both compartments with stirring for 5 min using a stir plate for planar bilayer recordings (SPIN-2, Warner Instruments).

2.4. Current recordings across planar lipid bilayers

For current recordings, we used amplifiers in voltage clamp mode (either Geneclamp-500 amplifier from Axon Instruments, or a BC-535 amplifier from Warner Instruments). The compartment of the bilayer chamber that was connected to the amplifier head stage by a Ag/AgCl electrode is referred to as cis compartment, the other one as trans compartment. We monitored the ionic current across the planar bilayers with a filter cutoff frequency of 10 kHz of the 4-Pole Bessel filters and recorded currents using LabVIEW 7.1 and an A/D converter card from National Instruments (PCI-6221) with a sampling frequency of 50 kHz. For analysis, data were filtered with a Gaussian filter at 100 Hz and analyzed by using the software Clampfit 9.2 (Axon Instruments). We determined all single-channel conductance values from the slope of current versus voltage (IV) curves, which were linear up to voltages of \(\pm 100\text{ mV}\) (see Supplementary data, Fig. S1). Ion channel recording was repeated at least 3 times for each experimental condition. We monitored the capacitance of the bilayers throughout the recordings using the built-in capacitance compensation of the amplifiers. For the determination of the estimate of the number of BTA-EG4 molecules in each self-assembled pore, we used time-averaged currents over discrete time intervals (3 min) as a function of increasing concentrations of BTA-EG4. We averaged transmembrane currents at positive and negative voltages at \(\pm 50\text{ mV}\) by integrating areas under current versus time traces and dividing the resulting area by the total recording time followed by normalizing the averaged currents by the membrane capacitance. All experiments were performed in 1.0 M or 3.0 M CsCl with 10 mM HEPES, pH 7.4 unless otherwise noted.

2.5. Statistical analysis of differences in single-channel conductance

To determine statistical significance of differences in single-channel conductance of BTA-EG4 or gA at different pH values, we compared all single-channel conductances obtained from different applied voltages.

We determined \(P\)-values using a two-sample Student’s \(t\)-test included in the Origin 8.0 software package (Northampton, MA).

2.6. Liposome leakage assay

We prepared liposomes from a total lipid concentration of 10 mM of the following lipids, DiPhyPC:cholesterol:DiPhyPG:DPPE-PEG\(_{2000}\) in a 9:9:1:1 molar ratio. We obtained a lipid film by placing the lipid mixture dissolved in chloroform in a 10 mL round bottom flask, followed by rotary evaporation. We rehydrated the lipid film with 750 \(\mu\text{L}\) of 20 mM HEPES, pH 5.5, containing a mixture the pH-sensitive dye fluorescein sulphonate (Molecular Probes, Eugene, OR) and the pH-insensitive dye, sulforhodamine B (Molecular Probes, Eugene, OR) at a concentration of 200 \(\mu\text{M}\) and 10 \(\mu\text{M}\), respectively, and incubated the liposomes for 4 h at 37 °C. The resulting lipid vesicles were subjected to 15 freeze-thaw cycles and extruded through 100-nm membrane for 21 times. We used Sephadex-G25 M gel filtration columns (GE Healthcare, Buckinghamshire, UK) to separate liposomes with encapsulated fluorescent dyes from the fluorescent dyes in solution, and to change the solution outside the liposomes to 20 mM HEPES, pH 7.4. We transferred the resulting liposomes to 96-well plates (100 \(\mu\text{L}\) in each well) followed by addition of compounds of interest. We monitored the fluorescence signal due to leakage of \(\text{H}^+\) ions every 5 min for 5 h with an excitation wavelength of 485 or 560 nm and an emission wavelength of 518 or 590 nm for fluorescein sulphonate or sulforhodamine B, respectively using a Fluroskan Ascent FL plate reader (Thermo Fisher Scientific, Waltham, MA). Increasing fluorescence signals indicated an increasing \(\text{pH}\) inside the liposomes, due to leakage of protons out of liposomes.

2.7. Cell viability assay

SH-SY5Y human neuroblastoma cells (ATCC) were maintained in Dulbecco’s Modified Eagle’s medium (DMEM)/F-12 (1:1) containing 10% (v/v) fetal bovine serum, 4 mM Glutamax, 100 U mL\(^{-1}\) Penicillin and 100 \(\mu\text{g}\) mL\(^{-1}\) Streptomycin in 5% \(\text{CO}_2\) at 37 °C. All culture reagents were purchased from Gibco Life Technologies (Carlsbad, CA). We seeded cells in a 96-well plate (50,000 cells well\(^{-1}\)) 24 h before performing cell viability assays. The cells were exposed for 24 h to reagents, which were diluted to the final concentration in Opti-MEM, a serum-free medium. We determined the cell viability using an MTT cell proliferation assay, according to the instructions from the supplier [52].

2.8. Microbial toxicity

\textit{E. coli} and \textit{B. subtilis} were grown in LB media overnight at 37 °C and 31 °C, respectively. The cells were harvested, washed, and subsequently resuspended in fresh LB medium containing compounds of interest at various concentrations. We added 200 \(\mu\text{L}\) of cells to each well of 96-well plates with three replicates for each condition and allowed the cells to grow for 30 h while shaking. We monitored the growth of bacteria by measuring optical density at 600 nm relative to control cells every 15 min, using a VersaMax microplate reader ( Molecular Devices Inc., East Falmouth, MA).

2.9. Hemolysis assay

We purchased bovine red blood cells (bRBC) from Lampire Biological Laboratories (Pipersville, PA). After washing bRBC in phosphate buffered saline (PBS) buffer (Dulbecco’s PBS, Gibco) at pH 7.4, we prepared the stock bRBC suspension by diluting the originally purchased suspension 10-fold in PBS buffer (after several wash steps in PBS). We pipetted 200 \(\mu\text{L}\) of the resulting bRBC stock solution into reaction vials and added various final concentrations of BTA-EG4 in PBS such that the final volume of all vials was 1 mL. These suspensions
were mixed and incubated at 37 °C for 1 h followed by centrifugation at 10,000 × g for 5 min at a temperature of 21 °C. To assess hemolysis, we collected the supernatant from each tube and determined the absorbance at 541 nm. We added 800 μL PBS as the negative control, which yielded 0% hemolysis and 800 μL diH2O as the positive control, which yielded 100% hemolysis. We performed the experiment in triplicates for each condition.

2.10. Measurement of fluorescence emission spectra

We diluted BTA-EG4 molecules to a final concentration of 50 μM in deionized H2O, pure octanol, and a liposome suspension. The liposomes were prepared from a total lipid concentration of 10 mM of DiPhyPC in water by the gentle dehydration rehydration method, followed by tip sonication. We transferred 800 μL of each BTA-EG4 sample to a disposable cuvette (Precision Cells, Inc., Farmingdale, NY), and measured fluorescence emission spectra at an excitation wavelength of 350 nm, using a Fluorolog-3 spectrophotometer (Horiba-Jobin Yvon, Edison, NJ).

3. Results and discussion

3.1. BTA-EG4 molecules self-assemble to well-defined pores with quantized conductance levels

Fig. 1 shows that BTA-EG4 molecules were capable of forming ion channels with well-defined single-channel conductance steps at concentrations of 10 μM or above (Fig. 1B). In contrast, the reactants for the synthesis of BTA-EG4, BTA or tetra-ethylene glycol (EG4), did not form detectable ion channels at concentrations up to 100 μM in planar lipid bilayers. Remarkably, self-assembly of BTA-EG4 appeared to form one predominant pore structure; only a few single channel events resulted in a single-channel conductance that was different from the predominant one (Fig. 1B) and for an extended current versus time trace, see Supplementary data, Fig. S2). These sub-conductances typically had exactly half the current amplitude of the main conductance (see Fig. 1B second channel opening event from the left). The probability for observing these sub-conductances was approximately one fifth of that for the main conductance and their lifetime was significantly shorter than that of the main conductance (Fig. S2). These sub-conductances may indicate a pair-wise organization of BTA-EG4 pores, in which the activity of each pore is usually stable compared to the sub-conductance state. Occasionally, we observed conductance values that were multiples of the amplitude of the main conductance value; we attribute these events to the simultaneous opening of two or more pores in the bilayer. From recordings at various applied potentials, we determined an average single-channel conductance of BTA-EG4 of 37.8 ± 1.3 pS in an electrolyte solution containing 1.0 M CsCl with 10 mM HEPES, pH 7.4. Recordings of the single-channel conductance through BTA-EG4 pores as a function of the concentration of CsCl revealed that the CsCl concentration required to reach half-maximal single-channel conductance [53] was 0.06 M (see Supplementary data, Fig. S3), and the maximum conductance was \( \gamma_{\text{max, Cs}} = 40.0 \pm 1.7 \) pS.

Table 1 compares single-channel conductance values of BTA-EG4 pores to those of gramicidin A (gA) pores under the same experimental conditions. The single-channel conductance of BTA-EG4 was only slightly smaller than that of gA, indicating that the inner diameter of pores from BTA-EG4 was probably on a similar size scale as the diameter of gA pores (i.e., ~0.4 nm) [54]. This comparison assumes that the spatial arrangement of polar groups in the lumen of BTA-EG4 pores was comparable to gA pores (see below) and that pores from BTA-EG4 had approximately the same length as gA pores. Given the molecular dimensions of BTA-EG4 (Fig. 1A), it is unlikely that pores from this molecule would be longer than the thickness of a lipid bilayer (i.e., ~3.5 nm) and it is also unlikely that these transmembrane pores would be significantly shorter than gA pores (i.e., ~2.6 nm) [55] since gA pores are already sufficiently short to induce a hydrophobic mismatch that leads to energetically unfavorable thinning of the lipid bilayer next to the opening of a gA pore [56,57].

3.2. Self-assembled pores from BTA-EG4 are selective for monovalent cations

Based on the small single-channel conductance of pores from BTA-EG4, we investigated the ion selectivity of these pores. Table 1 shows that BTA-EG4 pores were selective for monovalent cations, while divalent Ca2⁺ cations did not result in detectable single channel events. We were, however, able to observe macroscopic currents in an electrolyte containing 1.0 M CaCl2, if the concentration of BTA-EG4 exceeded 35 μM. When comparing various cations, the slopes from current versus voltage curves revealed the following order of conductance through BTA-EG4 pores: \( H^+ >> Cs^+ >> K^+ >> Na^+ >> Li^+ >> Ca^{2+} \). This order is exactly the same as in gA pores and it is close to the mobility sequence for these ions in water [58]. Based on these findings and on the observation that the absolute values of the single conductances in electrolytes from these different cations are also similar to those of gA pores, we propose that BTA-EG4 channels, like gA channels, behave essentially like water-filled pores [58].

In an electrolyte containing 1.0 M HCl (pH –0), we observed large single-channel conductances through BTA-EG4 pores that ranged from 200 to 800 pS, but the formation of open pores was less frequent than in less acidic electrolytes. For instance, in an electrolyte with 0.01 M HCl (pH ~2), pore formation by BTA-EG4 was more frequent than at pH 0 and its single-channel conductance did not fluctuate more than in electrolytes with a pH value of 7.4. These experiments revealed that the single-channel conductance of BTA-EG4 in 0.01 M HCl was comparable to its single-channel conductance in 1.0 M CsCl, indicating very high permeability of protons through these pores; a result that is consistent with a water-filled pore.

With regard to anions, monovalent potassium salts with large anions generated a slightly larger single-channel conductance through pores of BTA-EG4 in this sequence: KBr >> KNO3 >> KCl >> KF, although the maximum difference of the single-channel conductance between electrolytes containing KBr and KF was only 24% and this difference was not statistically significant at a significance level of 0.05.
the bulk conductance of aqueous solutions with various potassium salts, see Supplementary data, Table S1).

Based on the observation that the single-channel conductance, and in particular, the ion selectivity of BTA-EG4 pores, are very similar to that of gA pores, we propose that the mechanism of ion selectivity may also be similar. In gA pores, the selectivity filter is lined by the carbonyl oxygen atoms of the peptide backbone and cation selectivity results from interactions of this polar backbone with cations [53,59]. In BTA-EG4 pores, the polar ethylene glycol oxygen atoms could serve as the selectivity filter by lining parts of the lumen of the pore. Another mechanism that may contribute to the cation selectivity of BTA-EG4 pores is cation–π interaction of cations with the electron-rich π system of the BTA moiety. In this case, the interaction of the BTA group with small cations (e.g. Li⁺) would be stronger than with large cations (e.g. Cs⁺), leading to the observed reduction of single-channel conductance with decreasing size of monovalent cations [26,60,61]. It is also possible that both mechanisms contribute to the strong cation selectivity of BTA-EG4 pores.

3.3. Self-assembly of BTA-EG4 forms long-lived, open pores in lipid bilayers

Fig. 1C shows the lifetime of open channels from BTA-EG4, which was approximately twice as long (τ = 1.97 ± 0.08 s) as that of gramicidin A pores (τ = 0.95 ± 0.02 s) under the same experimental conditions (see Supplementary data, Fig. S5A). The long lifetime of pores from BTA-EG4 suggests either that the driving forces for the assembly of BTA-EG4 pores are stronger than the hydrogen bonding between the two hemi-channels in gA [53,62,63], or that the driving forces for the disassembly of BTA-EG4 pores are smaller than the hydrophobic mismatch that drives disassembly of gA pores [36,57,64].

3.4. Each BTA-EG4 pore contains approximately four monomers

In order to provide an estimate for the number of BTA-EG4 molecules in each self-assembled pore, we performed time-averaged recordings of transmembrane current over discrete time intervals as a function of the concentration of BTA-EG4 molecules [65]. Fig. 2 shows that increasing concentrations of BTA-EG4 resulted in a strong increase in transmembrane current. For instance, at concentrations above 35 µM BTA-EG4, multiple pores were opening at the same time, resulting in “macroscopic currents” across the membrane. By keeping the experimental conditions constant (e.g. constant applied voltage, ionic strength, type of electrolyte, bilayer thickness, etc.), we found that the time averaged currents and hence the macroscopic conductance, G, followed the relationship:

\[ G \propto [\text{BTA–EG}4]^n \] (1)

where n provides a rough estimate for the average number of monomers in the channels [65–67]. To account for possible variations in membrane area during an experiment or between different experiments, we normalized all time averaged currents by the membrane capacitance, which is proportional to membrane area and directly measurable [68]. This analysis suggests that pores from BTA-EG4 consisted of 4.2 ± 0.9 BTA-EG4 monomers (Fig. 2). For comparison, we performed a similar experiment with gA and obtained an estimate of 2.08 ± 0.08 monomers of gA in a single channel (see Supplementary data, Fig. S4B), which is in agreement with pore formation by dimerization of two gA monomers [62].

Fig. 2 shows that the self-assembly of BTA-EG4 to pores in planar lipid bilayers required relatively large concentrations (≥10 µM), while gA is capable of forming pores at approximately one million-fold lower concentrations (see Fig. S4B). We attribute this difference mainly to the extremely low solubility of gA in aqueous solutions; at equilibrium gA partitions almost completely into the bilayer phase. BTA-EG4, in contrast, is significantly more water soluble than gA and therefore it does not concentrate in the bilayer as much as gA. Moreover, self-assembly of tetrameric BTA-EG4 pores may require a larger local concentration of BTA-EG4 molecules in the bilayer than self-assembly of dimeric gA pores. In the context of a possible use of BTA-EG4 as a starting molecule for the synthesis of pore-forming antibiotics, this requirement for micromolar concentrations is a disadvantage compared molecules such as gA.

On the other hand, BTA-EG4 meets the criteria of Lipinski’s empirical rule of five [69] and small chemical modifications may increase its lipophilicity and potency while preserving its pore-forming activity.

3.5. The noise level of open BTA-EG4 pores is larger than that of open gA pores

In order to assess the conformational stability of BTA-EG4 pores, we compared the current noise through the main open state of BTA-EG4 pores with the noise through single gA pores. For this analysis, we used original current traces that were hardware-filtered with a four-pole Bessel filter with a cutoff frequency of 10 kHz and sampled at 50 kHz. Based on eight different open events of the main open state of BTA-EG4 pores, we found an average rms current noise (i.e., average standard deviation of the current from the mean current) of 0.650 ± 0.033 pA. For comparison, the average rms current noise from nine different opening events of individual gA pores was 0.277 ± 0.052 pA, when determined in the same way and under identical experimental conditions as for BTA-EG4 channels. Note, these noise values represent the average rms current noise through the respective baseline noise values represent the average rms current noise through the respective baseline noise from the recording setup (i.e., the baseline noise when no channel was open) and the current noise from fluctuations of ion flux through an open ion channel were both random and independent of each other. Since current histograms from the open events of BTA-EG4 and gA channels could be fitted well with Gaussian distributions (Fig. 1B), the assumption of randomness appears justified. Finally this simplified approach also assumes that differences in frequency-dependence of these two noise sources can be neglected.

Based on these assumptions, two-sample t-tests revealed that the average rms current noise through open BTA-EG4 pores was significantly larger than through open gA pores (p<0.01). Given that a
part of this rms current noise likely resulted from small conformational fluctuations of the channels [70], this difference suggests that these fluctuations were more prominent in open pores of BTA-EG4 than of gA. This result may on the one hand be surprising since the lifetime of BTA-EG4 pores is longer than that of gA pores, on the other hand, BTA-EG4 pores contain approximately four monomers that can each undergo small fluctuations in an open pore, while gA pores contain only two monomers.

3.6. The single-channel conductance through BTA-EG4 pores has a minimum at pH 3

In order to gain insight in the arrangement of BTA-EG4 molecules to a pore, we characterized the pH dependence of the single-channel conductance through these pores. Since BTA-EG4 contains an anilinium group with a pKa of approximately 4.6 in pure water [71,72], we hypothesized that protonation of this group in acidic electrolytes may reduce the permeation of cations through these pores by electrostatic repulsion. Fig. 3 shows that the single-channel conductance of BTA-EG4 pores had a minimum at a pH ~3. Since the high concentration of CsCl (3.0 M) in the electrolyte used for these recordings might have shifted the pKa of the anilinium group toward a more acidic pKa [73], we determined the pKa of BTA-EG4 in the presence of 3.0 M CsCl (and 12% DMSO to increase the solubility of BTA-EG4 and to mimic the hydrophobic environment in the proximity of planar lipid membranes). Under these conditions, an acid–base titration experiment indicated a pKa of ~3.5 for BTA-EG4. This pKa value is consistent with protonation of BTA-EG4 molecules and with concomitant reduction of the single-channel conductance of cations through pores from BTA-EG4 molecules at pH values below 4. Since the Debye screening length in this 3.0 M CsCl electrolyte was approximately 0.2 nm [73], the minimum conductance of BTA-EG4 pores at a pH value of ~3 suggests that the aromatic ring with the protonated anilinium group was located either at the mouth of the pores or faced the lumen of the pores. In contrast to the reduction of the single-channel conductance as the pH in the electrolyte was lowered from 7.4 to 2.8, the increase in conductance at pH values below 2.8 was likely due to the increased concentration of highly permeable protons in these increasingly acidic electrolytes (see Table 1, and Supplementary data, Fig. S5).

For comparison, Fig. 3 shows data from gA pores, whose conductance increased steadily with increasing acidity in the same 3.0 M CsCl electrolyte, indicating that the observed minimum conductance of BTA-EG4 pores at pH ~3 is characteristic for these pores and likely a consequence of protonation of the anilinium group of BTA-EG4.

3.7. Fluorescence spectroscopy suggests localization of the BTA moiety inside lipid bilayers

To gain complementary insight in the assembly of BTA-EG4 molecules to pores, we took advantage of the dependence of the fluorescence emission spectra of BTA derivatives on their chemical environment. Excitation at 350 nm revealed a shift to shorter emission wavelengths when BTA-EG4 molecules were dissolved in octanol compared to water. The wavelengths of maximum emission of BTA-EG4 in an aqueous suspension of liposomes was between the wavelength of maximum emission in water and octanol, suggesting that at least a part of the fluorescent BTA moiety was located inside the bilayer membrane (Fig. 4).

3.8. Asymmetric addition of BTA-EG4 to one side of the bilayer leads to symmetric current–voltage relationships

In order to test whether membrane incorporation of BTA-EG4 molecules from only one side of the bilayer would lead to pores with transverse asymmetry across the bilayer [74], we obtained a current versus voltage curve after addition of BTA-EG4 only to the cis-compartment. Since the resulting current versus voltage curve was linear with the same slope at both polarities, we did not observe a measurable indication of transverse asymmetry of BTA-EG4 pores in DiPhyPC bilayers (see Supplementary data, Fig. S1).

3.9. High concentrations of BTA-EG4 molecules destabilize lipid bilayers

At concentrations above 35 μM, BTA-EG4 occasionally destabilized planar bilayers sufficiently to break the membranes irreversibly. We observed this effect in approximately 20% of experiments. In addition, liposomal leakage assays revealed that BTA-EG4 induced leakage of protons through liposome membranes (see Supplementary data, Fig. S6). The same liposome-based assay revealed that EG4 by itself did not cause detectable leakage, while BTA by itself was capable of inducing approximately half the rate of proton leakage than BTA-

![Fig. 3. Single-channel conductance of BTA-EG4 and gramicidin A as a function of the pH in the recording electrolyte. Ion channel recordings were performed with DiPhyPC bilayers in unbuffered electrolytes that contained 3.0 M CsCl at various pH values that were adjusted by adding HCl. Statistical significance (p < 0.05) of differences in single-channel conductance of BTA-EG4 pores at different pH values was determined with respect to the minimum conductance at pH 2.8 and indicated by an asterisk (*). Error bars represent the standard error of the mean.](image)

![Fig. 4. Comparison of fluorescence emission spectra of 50 μM BTA-EG4 in pure H2O (■), pure octanol (●), and in an aqueous liposome suspension (▲) using an excitation wavelength of 350 nm. Liposomes were made of DiPhyPC with a total lipid concentration of 10 mM in water.](image)
EG. We could not, however, detect any ion channel activity from BTA by itself (or from EG4 by itself) in planar ion channel recordings.

3.10. **BTA-EG4 has antibacterial activity**

Gramicidin A and its analogs have antibiotic activity by forming cation-selective pores in biological membranes [59,63,75]. This activity is directed against gram-positive bacteria, not gram-negative bacteria, due to differences in structure of the bacterial cell walls [76,77]. Since we observed similarities in the characteristics of pores from BTA-EG4 and gA, we tested the antibacterial activity of BTA-EG4 molecules on a gram-positive and a gram-negative bacterium. Fig. 5 shows that BTA-EG4 inhibited the growth of the gram-positive bacterium *B. subtilis*: a concentration of 100 μM BTA-EG4 completely suppressed the growth of these bacteria after overnight exposure. The extent of this inhibitory effect was similar to that of 10 μM kanamycin or 5 μM gA, as shown in Fig. 5 of the Supplementary data. Consequently, we estimated a MIC value (i.e., the minimal concentration that inhibited visible growth over night) of ~100 μM BTA-EG4, while its IC50 value was ~50 μM. For comparison, we found the following values for five other pore-forming, antimicrobial peptides against gram-positive bacteria: i) alamethicin, IC50 = 1–3 μM [78]; ii) gramicidin D, MIC = 0.5 μM; iii) melittin, MIC = 8.5 μM; iv) magainin II, MIC = 18 μM; and v) human defensing HNP-1, MIC >60 μM [79]. In contrast, BTA-EG4 concentrations up to 500 μM did not show significant toxicity on gram-negative *E. coli* bacteria (see Supplementary data, Fig. S8).

3.11. **BTA-EG4 is toxic to human neuroblastoma cells**

Since BTA-EG4 was capable of forming ion channels in artificial lipid bilayers and exhibited antibacterial activity against gram-positive bacteria, we investigated the effect of BTA-EG4 on the viability of a human neuroblastoma cell line. After treating SH-SY5Y cells for 24 h in serum-free media containing various doses of BTA-EG4, we determined their viability by an MTT assay [52]. Fig. 6 shows that BTA-EG4 was toxic to these mammalian cells in a dose-dependent manner with an IC50 value of ~60 μM. For comparison, the IC50 value of gA in human embryonic kidney (HEK) cells was ~0.12 μM.

Since BTA-EG4 concentrations above 35 μM occasionally broke planar lipid bilayers irreversibly and since the IC50 values for killing gram-positive bacteria and neuroblastoma cells were above this concentration, we tested whether BTA-EG4 exerted its cytotoxic activity by cell lysis or by pore formation. To this end, we performed a hemolysis assay by exposing red blood cells to increasing concentrations of BTA-EG4. This assay revealed that BTA-EG4 does indeed have hemolytic activity, which, at BTA-EG4 concentrations above 200 μM, can reach 80% of the maximum hemolytic activity (Fig. S9). The concentration to reach half-maximal hemolytic activity (~140 μM), however, was significantly higher than the IC50 values of ~50 and ~60 μM for killing bacteria or neuroblastoma cells. In fact, below a concentration of 80 μM, BTA-EG4 had no detectable hemolytic activity. Together, these findings suggest pore formation with concomitant disruption of ionic gradients as a possible mechanism of toxicity of BTA-EG4 molecules. Similar to gA molecules, the large conductance to protons may cause BTA-EG4 molecules to act as an uncoupler of oxidative phosphorylation [80,81].

**Fig. 5.** Inhibitory effect of BTA-EG4 on the growth of *Bacillus subtilis* bacteria 22 h after incubation in LB media containing various concentrations of BTA-EG4. Growth was quantified by the optical density at a wavelength of 600 nm relative to untreated control cells. The concentration of BTA-EG4 molecules that inhibited growth by 50% (IC50 value) was ~50 μM. Each point represents the mean of two experiments with three replicates in each experiment; error bars represent the standard error of the mean.

**Fig. 6.** Cytotoxicity of BTA-EG4 on human neuroblastoma cells (SH-SY5Y) 24 h after exposure. Each point represents the mean of two or three experiments with six replicates in each experiment. Error bars reflect the standard error of the mean. The red curve is a fit of the equation, \( y = A_2 \left(1 - \frac{1}{1 + \frac{X}{IC_{50}}}ight) \) to the data on a linear x-scale, where \( A_1, A_2, \) and \( h\) are constants. The IC50 value was ~60 μM.

4. **Conclusion**

Self-assembly of a tetra-ethylene glycol derivative of benzothiazole aniline in planar lipid bilayers led to well-defined and long-lived transmembrane ion pores that are strongly selective for monovalent cations. In contrast to pores formed by gramicidin A, pores from BTA-EG4 have a minimum single-channel conductance at pH ~3, which suggests that the protonatable anilinium group is located less than 2 Å away from the lumen of the pores. Likely due to its pore-forming ability, this molecule exhibits antibiotic activity against gram-positive bacteria with an IC50 value of ~50 μM, but not against gram-negative bacteria. As expected for a pore former, BTA-EG4 was also toxic to human neuroblastoma cells with an IC50 value of approximately 60 μM. Given the recent development of molecules with similar general structure (i.e., a short hydrophobic/aromatic component attached to a short OEG group) as in vivo diagnostic agents for Alzheimer’s disease [48,50], our findings highlight the risk of pore formation as a mechanism for possible cytotoxic side effects. On the other hand, this small synthetic pore-forming molecule might be appealing as a starting material for the development of synthetic ion channels, antibiotics, membrane-permeating agents for drug delivery, or as uncouplers of oxidative phosphorylation since it is able to cross cellular membranes [50] and since oligo(ethylene glycol) moieties are biocompatible and suitable for polymeric drug delivery [82].
Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jbiolam.2011.08.024.

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