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Self-Assembled Nanochannels

Triazole-Tailored Guanosine Dinucleosides as Biomimetic Ion Channels to Modulate Transmembrane Potential

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Dedicated to Professor Dr. Goverdhan Mehta on the occasion of his 70th birthday.

Abstract: A "click" ion channel platform has been established by employing a clickable guanosine azide or alkyne with covalent spacers. The resulting guanosine derivatives modulated the traffic of ions across the phospholipid bilayer, exhibiting a variation in conductance spanning three orders of magnitude (pS to nS). Förster resonance energy transfer studies of the dansyl fluorophore with the membrane binding fluorophore Nile red revealed that the dansyl fluorophore is deeply embedded in the phospholipid bilayer. Complementary cytosine can inhibit the conductance of the supramolecular guanosine channels in the phospholipid bilayers.

Biological membranes consisting of a lipid bilayer play fundamental roles in partitioning cells and organelles in all living organisms. Transmembrane proteins embedded in the bilayer, that is, natural ion channels, facilitate the transport of ions across these highly insulating barriers.[1] Inspired by these channels in living systems, the creation of natural and non-natural molecules that can mimic structural aspects of natural transmembrane ion channel proteins in lipid bilayers have received much attention in recent years.[2,3] Biomimetic nanochannels have been developed to understand mechanistic details of channel proteins on the molecular level[4] and have been found useful as drug delivery systems,[5] antimicrobial agents[6] and biosensors.[7] Most nonpeptidic ion channels have been prepared using multistep linear synthesis with low overall yield.[2–4] The development of a facile and modular synthetic approach would enable the creation of a large array of membrane active structures for studying the structure–activity of a class of compounds. The key features of synthetic ion channels that need to be addressed are the pore size, ion selectivity, voltage and ligand gating and blockage of the channels by using specific compounds.

Recently lipophilic nucleoside derivatives have been designed to create complex self-assembled structures with desirable function.[8] Among the nucleobases, guanine has generated wide interest in various areas of research ranging from molecular biology to nanotechnology.[9] Guanosine derivatives can self-associate to form cyclic tetramers called G-quartets, which are planar arrangements of four guanine molecules linked together by Hoogsteen-type hydrogen bonds.[10a,c] These quartets stack on top of one another to give a columnar aggregate, which is known as a G-quadruplex motif and is stabilized by certain cations, for example, Na^+, K^+. These supramolecular structures are believed to play a key role in the biology of cancer and ageing.[10d–f] As a hydrogen-bonded macrocyclic arrangement with ionophore properties, G-quartets signify a promising scaffold for fabricating synthetic ion channels.

Sakai et al. reported that folate dendrimers containing a similar hydrogen bonding unit like guanine form synthetic transmembrane ion channels with a conductance of 21 pS.[11] Davis and co-workers reported that ditopic guanosine–bile acid conjugates can form ion channels in phospholipid membranes with nanosiemens (nS) conductance.[12] We envisioned that if a modular synthetic strategy based on Cu^I-catalyzed 1,3-dipolar azide–alkyne cycloaddition can be developed, it would significantly expand the structure–function relationships of the guanosine-based ion channels through tethering of the covalent spacers between two guanosine units. Herein, we report the design and synthesis of diguanosine derivatives using two efficient and modular strategies based on "click chemistry"[13] between either a clickable guanosine azide or an alkyne with aromatic, amphiphilic and lipophilic linkers. The ion channel activity of these guanosine derivatives have been demonstrated using voltage-clamp experiments, which show that diguanosine derivatives form discrete channels with stable and large pores with nS conductance in the phospholipid membrane.

Following extensive optimization, we have developed two general approaches for synthesizing the acetylene and azide building blocks (Figure 1, Scheme 1 and Scheme 2). We have incorporated: 1) the azide unit in guanosine **1**, and 2) the acet-

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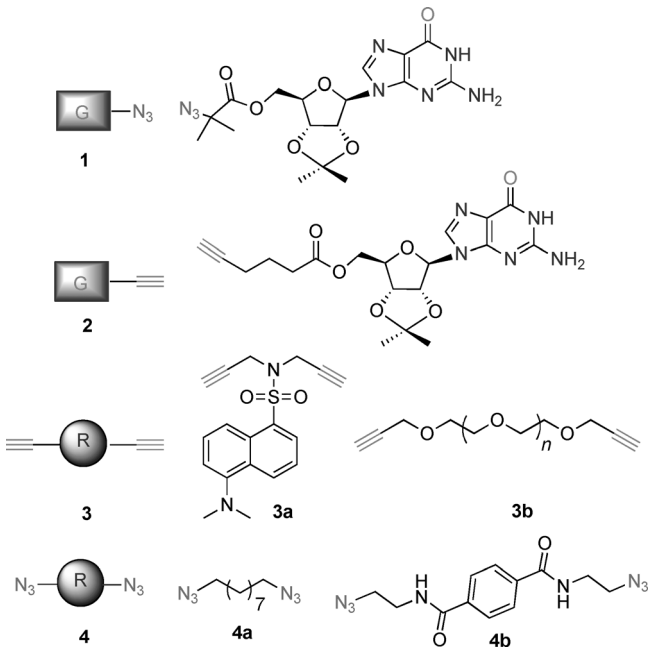
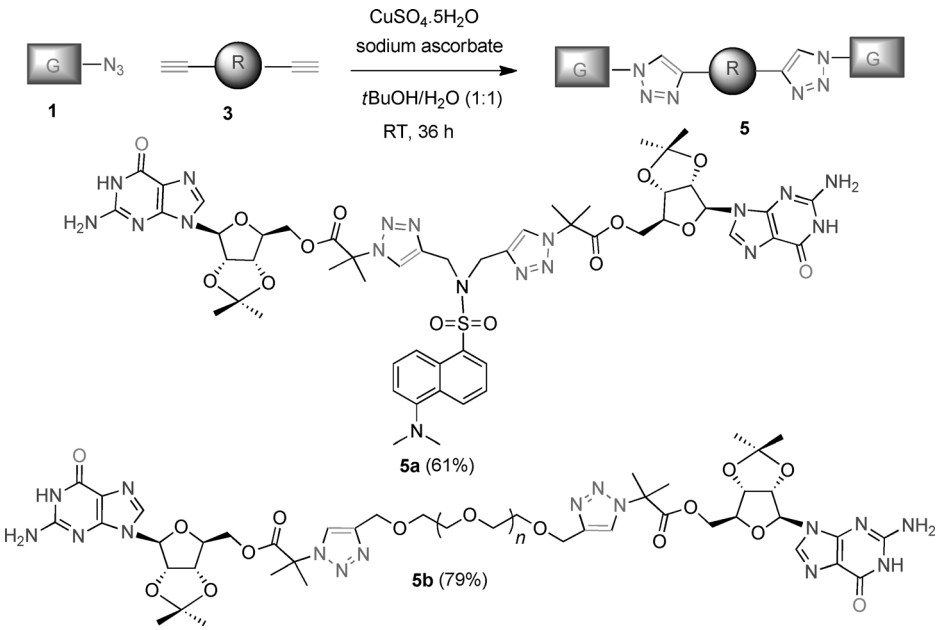


Figure 1. Azide and alkyne building blocks.



Scheme 1. Synthesis of bis-guanosine derivatives from azido guanosine.

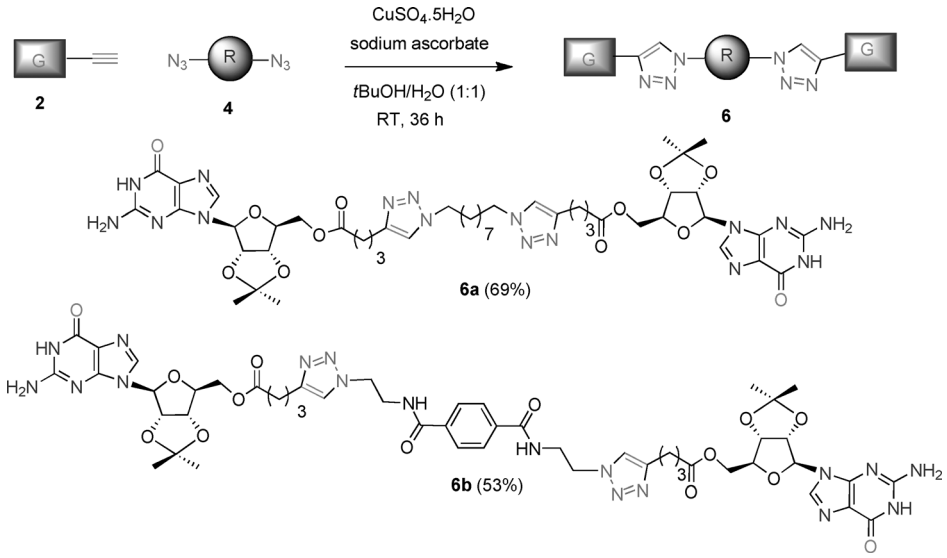
ylene unit in guanosine **2** (Scheme 1 and Scheme 2). The clickable lipophilic guanosine azide **1** and guanosine alkyne **2** building blocks were prepared from guanosine using two to three step protocols (see the Supporting Information). The fluorescent dansyl dialkyne **3a** was prepared from the commercially available dansyl amide in high yield (see the Supporting Information). The azido guanosine derivative **1** was treated with the dansyl containing dialkyne **3a** in the presence of Na-ascorbate and $CuSO_4{\cdot}5H_2O$ in *t*BuOH/H_2O (1:1) as solvent[14] to give fluorescent diguanosine nucleoside **5a** in high yield.[15]

The fluorescent dinucleoside **5a** can self-assemble to provide a G-quartet-based fluorescent ion channel, and the ion channels can be stabilized due to the π–π interactions between the naphthalene rings of the dansyl derivative of each G-quartet layer. Then, we introduced a polyethylene glycol (PEG) linker between two guanosine units by ligation of PEG diacetylene **3b** ($n=9$) with the guanosine azide **1** under standard "click" condition. The ditopic guanosine **5b** containing biocompatible PEG as a spacer can provide hydrophilicity to the hydrophobic guanosine nucleoside and may lead to the formation of G-quartet-based amphiphilic ion channels.

The second approach was then followed to access lipophilic and carboxamide-containing guanosine derivatives (Scheme 2). Click reaction was performed between two lipophilic guanosine alkyne **2** with a lipophilic polyalkane diazide **4a** to obtain the bis-guanosine triazole derivative **6a** in high yield (Scheme 2). An aromatic bis-carboxamide azide **4b** was successfully employed as the spacer with the guanosine alkyne **2** under identical reaction condition to afford **6b** in good yield (Scheme 2).

To demonstrate that these dinucleosides form ion channels for K^+ ions, voltage-clamp experiments on planar solvent-free bilayers were performed in a buffer containing KCl (1 M) and NaH_2PO_4 (2 mM) at pH 7.4. Planar solvent-free bilayers were generated by spreading giant unilamellar vesicles (GUVs) consisting of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) and cholesterol (9:1; Figure S1 in the Supporting Information) on a small aperture in glass.[16] Current traces were recorded after adding the dinucleoside **5** and **6** (20 μM) to the cis-side of the chamber at an applied voltage of −100 mV (Figure 2, Figures S2–S4 in the Supporting Information).

The insertion of nucleosides **5** and **6** into the lipid membrane resulted in characteristic current steps suggesting the formation of a defined ion channel (Figure 2) that opens and closes. The observed opening and closing can be attributed to the dynamic self-assembly and disassembly of the chiral supramolecular structures formed by the guanosine derivatives. Statistical data analysis of the current traces showed that there is a collection of conductance values spanning three orders of magnitude from <0.1 to 10 nS. Guanosine–dansyl conjugate **5a** exhibited 61.3% of the <0.1 nS channels and 36.1% of the 0.1–0.5 nS ones with lifetimes of up to 1 s. The diguanosine **5b** with the flexible PEG linker produced considerably larger conductance values within seconds, which is consistent with larger pores (Figure 2, Table 1).



Scheme 2. Cu^{I} catalyzed Huisgen 1,3-dipolar cycloaddition with a guanosine alkyne.

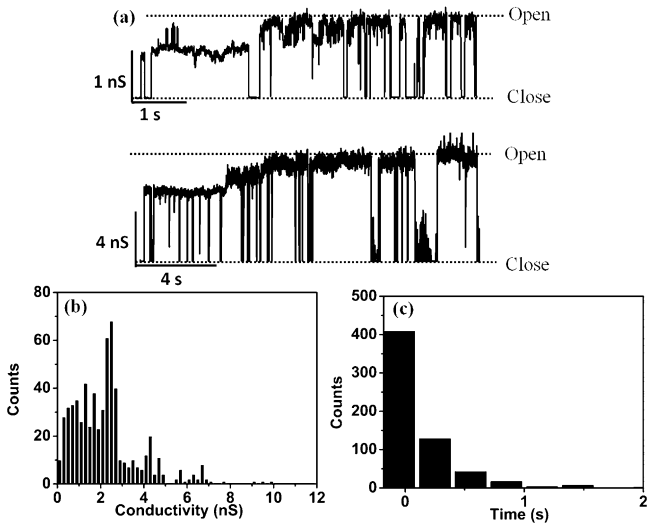


Figure 2. a) Results of voltage-clamp experiments showing representative states recorded after the addition of **5b** (20 μM) to the cis-side of the chamber after the planar bilayer was formed. The experiment was performed at −100 mV in a buffer containing KCl (1 M) and Na_2HPO_4 (2 mM) at pH 7.4. b) Distribution of the observed conductance states (628 events) of compound **5b**. c) Open time distribution (628 events) of **5b**.

Remarkably, about 48% of these channels formed by **5b** showed conductance values of 2–5 nS and are "open" up to 5 s in the planar membranes. Diguanosine **5b** also showed channels with conductance values of 5–10 nS (5.3%). It is noteworthy that crown ethers usually exhibit pS range conductance, whereas a PEG–diguanosine forms channels with nS conductance. The putative ion channel formed by **6a** containing lipophilic alkyl groups provided a conductance of 1–2 nS (54.8%). It was also observed that the channels formed by **6a** were more stable and "open" for a longer time compared to the hydrophilic polyether **5b** (Table S1 in the Supporting Information). This may be attributed to the higher lipophilicity of the species formed by **6a**. The diguanosine **6b** containing phenylene dicarboxamide unit as the spacer most frequently exhibited channels with conductance levels of 1–2 nS (87.6%).

We then investigated the ion channel activity of diguanosine–PEG conjugate **5b** using different ionic species passing through the membrane (Table 2). Since various monovalent cations of different size can template a wide variety of supramolecular structures, we hypothesized that the channel's conductance could alter in the presence of such monovalent cations.[9,10] The conductance depends on the pore size as well as on the size of the ions. A sufficiently large channel does not require full dehydration of the ion. It is interesting to note that small ion channel conduc-

Table 1. Frequency of events for channels formed by **5** and **6**.[a]

Conductance [nS]	**5a** events [%]	**5b** events [%]	**6a** events [%]	**6b** events [%]
<0.1	428 (61.3)	4 (0.6)	2 (0.3)	–
0.1–0.5	252 (36.1)	49 (7.9)	106 (15.1)	8 (0.9)
0.5–0.8	18 (2.6)	50 (8.0)	52 (7.4)	30 (3.3)
0.8–1	–	35 (5.6)	81 (11.5)	67 (7.4)
1–2	–	152 (24.4)	384 (54.8)	789 (87.6)
2–5	–	299 (48.0)	70 (10.0)	6 (0.7)
5–10	–	33 (5.3)	6 (0.9)	1 (0.1)
>10	–	1 (0.2)	–	–

[a] The number and frequency of ion channel open events were taken from patch-clamp experiments (Figure 2, Figures S2–S4 in the Supporting Information). Numbers in brackets indicate the relative percentage between the boundaries.

Table 2. Frequency of events for channels formed by **5b** in the presence of different ions.[a]

Conductance [nS]	Na^+ events [%]	K^+ events [%]	Cs^+ events [%]
<0.1	–	4 (0.6)	8 (1.3)
0.1–0.5	–	49 (7.9)	85 (14.1)
0.5–0.8	–	50 (8.0)	8 (1.3)
0.8–1	–	35 (5.6)	1 (0.2)
1–2	469 (22.6)	152 (24.4)	401 (66.4)
2–5	1591 (76.8)	299 (48.60)	98 (16.2)
5–10	12 (0.6)	33 (5.3)	3 (0.5)
>10	–	1 (0.2)	–

[a] The number and frequency of ion channel open events were taken from patch-clamp experiments (Figure 2, Figures S5 and S6 in the Supporting Information). Numbers in brackets indicate the relative percentage between the boundaries.

tance in the range of 0.1–0.8 nS was observed in K^+- and Cs^+-containing buffer, whereas a larger conductance of 1–5 nS with more events was observed in the presence of Na^+. Channels with conductance levels of 1–2 nS (22.6%), 2–5 nS (76.8%) and 5–10 nS (0.6%) were formed by **5b** in Na^+-containing buffer. The conductance decreased to 1–2 nS in Cs^+-containing buffer. These results indicate the presence of multiple pore structures in the membrane and the selectivity is controlled by the self-organized dynamic structure in the presence of different monovalent ions of different size. No measurable conductance was observed for **5b** in $MgCl_2$ and $CaCl_2$ in planar bilayer membranes indicating high selectivity for monovalent cations.

Circular dichroism spectroscopic analysis of **5b** provides evidence in support of G-quadruplex formation in the phospholipid bilayer (Figure S7 in the Supporting Information).[17] Fluorescence spectroscopy was then used to investigate the location of the dansyl group of **5a** in the phospholipid bilayer.[18,19] The fluorescence emission spectra of **5a** were recorded with an increasing concentration of liposomes ($\lambda_{ex}=350$ nm, Figure S8 in the Supporting Information). In the absence of liposomes, the dansyl–guanosine conjugate **5a** showed two emission bands at 430 and 554 nm, which were assigned to the guanosine and the dansyl group, respectively. The fluorescence intensity of guanosine band of **5a** increased after stepwise addition of liposome suspension, indicating binding of the ionophore **5a** to the membrane (Figure S7 in the Supporting Information).[20d] Further, the emission maximum of the dansyl group showed an about 19 nm blue shift with increasing liposome concentration (0–1 μM lipid).[20] This blue shift in emission maximum is characteristic for a less polar and less hydrated microenvironment of the dansyl group and can be interpreted as a localization of the dansyl fluorophore of **5a** in the bilayer.

Förster resonance energy transfer (FRET) studies further suggest that the dansyl fluorophore is localized in the phospholipid bilayer. Nile red is a membrane binding dye, the photophysics of which in lipid bilayers has widely been studied.[18,20] As the absorption maxima (551 nm) of Nile red overlaps with the emission of **5a**, these two can be considered as a donor–acceptor FRET pair, in which **5a** acts as a donor moiety, while Nile red is the acceptor. The results show that upon addition of **5a** to a Nile red containing vesicle suspension, the intensity of Nile red at 620 nm increases, whereas the intensity of **5a** at 535 nm decreases (Figure 3). Here, efficient FRET indicates that the dansyl moiety must be in close proximity to the Nile red molecule, which is well known to specifically bind to the lipid membrane.[20] The ion current steps (Figure 2, Figures S5 and S6 in the Supporting Information) can thus be attributed to an assembly and disassembly of stably inserted G-quartet aggregates and are most likely not related to transient insertions of molecules into the membrane.

The carboxyfluorescein (CF) release assay was used to monitor whether large pores are formed in the vesicles upon interaction with **5a** that might cause the observed large conductance values.[21] CF-encapsulating vesicles were prepared composed of DPhPC/cholesterol (9:1). The PEG–guanosine conjugate **5b** (100 μM in DMSO) was added to the vesicles preloaded with CF and the fluorescence intensity was monitored over time at 520 nm ($\lambda_{ex}=492$ nm). No release of CF was observed in the absence of **5b** or upon addition of DMSO. Addition of **5b** led to a small increase in fluorescence intensity, indicating that **5b** does not significantly alter the integrity of the membrane (Figure 4). The percentage of CF release was determined by monitoring the fluorescence intensity of released CF and compared to a 100% release by destroying the liposomes using Triton-X. The percentage release of CF from vesicles into the extravesicular solution was calculated to be 12.3% after 1 h.

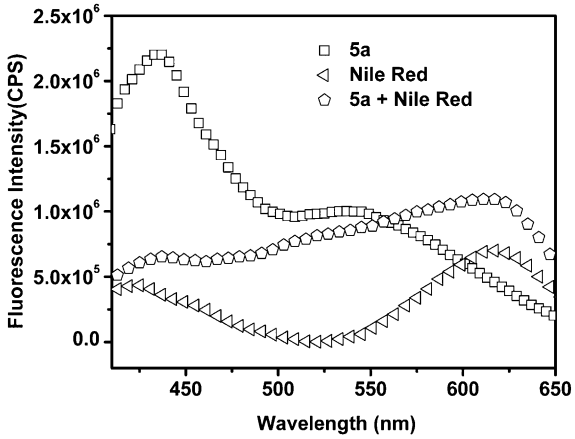


Figure 3. Fluorescence spectra ($\lambda_{ex}=350$ nm) of dansyl–guanosine conjugate **5a** (500 nM), Nile red (5 μM), **5a** and Nile red in the presence of LUVs in PBS (2.7 mM KCl, 136.9 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM NaH_2PO_4, pH 7.4).

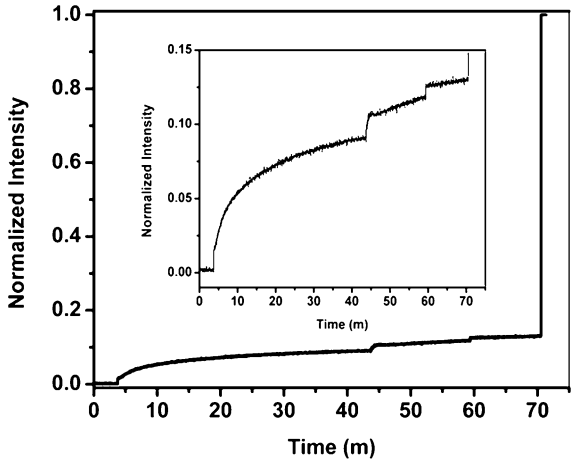


Figure 4. Time-course of CF leakage from liposomes (DPhPC/cholesterol, 9:1) caused by **5b** in PBS (2.7 mM KCl, 136.9 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM NaH_2PO_4, pH 7.4). At $t=70$ min, the liposomes were treated with Triton-X.

The dansyl–guanosine conjugate **5a** most frequently exhibited channels with small conductance values in the range <0.1 nS (Table 1), which can be attributed to the size of a G-quartet diameter similar to that reported for the folate dendrimer, which produced a conductance of 21 pS.[12] The π–π interactions and the hydrophobic interactions of the dansyl central unit may stabilize the supramolecular architecture of the quartets within the bilayer. Since the insertion of **5b** and **6** into

membranes produced large conductance in the nS range, the functional pores are expected to be larger than a quartet formed from each of **5** and **6**.[12–14] The voltage clamp experiments of dinucleosides **6a** and **6b** indicated the formation of thermodynamically favorable ion channels with a conductance range of 1–2 nS. Guanosine derivative **5b** containing the long chain spacer like PEG gave higher conductance. The self-association of the guanosine moieties into a G-quartet would result in a columnar supramolecular architecture to form barrel-stave-type ion channels (Figure 5).

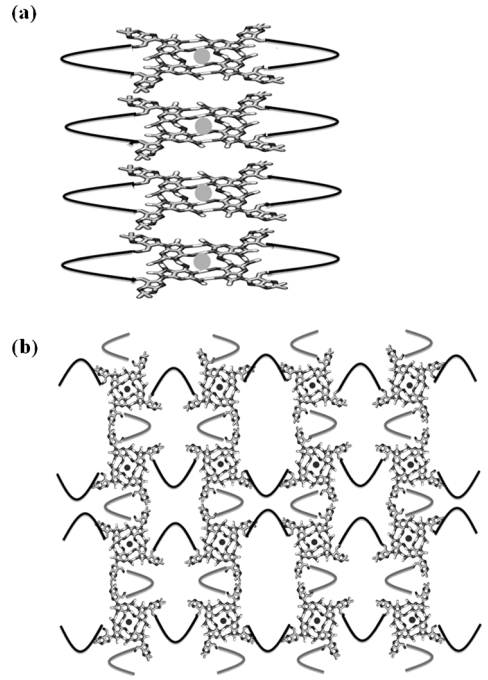


Figure 5. Proposed model of: a) barrel-stave-type cation channels, and b) formed by diguanosine derivatives in a phospholipid bilayer.

A cation-filled G-quadruplex presumably directs the assembly of these dynamic structures within the membrane. Since the size of the monovalent cations can lead to variations in the supramolecular organization of guanosine derivatives, different cations found to alter pore size and opening and closing of the channels. Since we did not observe significant leakage of CF (~10 Å long and 6.5 Å wide) from liposomes using the PEG derivative **5b**, we hypothesize the formation of barrel-stave-type of ion channels (Figure 5a). The channels formed by **5b** with conductance values of 5–10 nS (5.3%) are expected to form barrel-stave-type ion channels (Figure 5b) which can promote the transport of CF (12.3% after1 h).

Our next effort was to study the inhibition of the ion channels formed by the insertion of guanosine derivatives into the lipid bilayers. We envisaged that, since the ion channels are formed from the spontaneous self-assembly of guanosine, the addition of cytosine to the lipid bilayer would break the self-assembly of guanosine and inhibit the ion flow. Due to the complementary geometric arrangement of hydrogen-bond donors and acceptors in guanosine and cytosine, they can associate and the channels disappear. We observed that ion channels formed from compound **5b** disappear upon addition of excess cytosine (5 mM) to the lipid bilayers containing **5b**, which may be attributed to a lack of any stable (long-lasting) three-dimensional structure (Figure 6). CD spectra of a solution

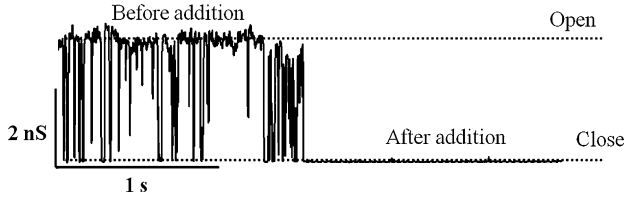


Figure 6. Inhibition of ion channel formed from **5b** using complementary cytosine.

containing **5b** (10 μM) and LUVs at pH 7.4 in PBS were recorded in the absence and presence of cytosine. Addition of 7 equiv of cytosine led to a decrease of the band intensity at 246 nm and formation of several new positive peaks at 261, 276 and 282 nm (Figure S9 in the Supporting Information). This suggests a structural transition upon addition of cytosine.[22]

In summary, a self-assembled "click" ion channel platform has been established using a one-pot azide–alkyne cycloaddition of lipophilic guanosine azide and guanosine alkyne building blocks with a variety of covalent spacers. Our results show that not only bile-acid units can be used as a spacer between guanosine units for transport of ions, but the pore size and conductance can be modulated to give more regular openings by changing simplified click spacers. The noteworthy aspects of our approach include expansion of guanosine-based ion channels, monitoring the formation of ion channels using fluorescent guanosine in the lipid bilayer and cytosine induced inhibition of the conductance formed from the PEG-linked guanosine. These investigations should enable a new modular approach for the preparation of analogues and decoration of existing ion channels leading to increased diversity and in turn, pore size and ionic selectivity.

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