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# Bis(sulfonamide) transmembrane carrier allows pH-gated inversion of ion selectivity

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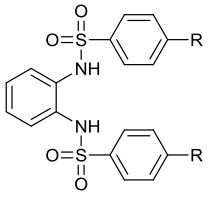
**Bis(sulfonamide) based synthetic carriers are reported for inversion of ion selectivity upon deviation of pH within a narrow window. A liposomal membrane potential is also generated when potassium ion is passively transported by these carriers.**

The bilayer lipid membranes of a cell act as a barrier and protect the cell from its external environment. They are impermeable to ions found in the extracellular and intracellular fluids, such as $Na^+$, $K^+$, $Ca^{2+}$, $Cl^-$, etc. Therefore, nature has developed many sophisticated membrane proteins which facilitate the transport of ions across membranes. This process is very crucial for maintaining the ionic and pH homeostasis of cells.[1] The ion and pH homeostasis are fundamental regulators of cellular processes, such as cell volume regulation, energy transduction, sensory perception, cell adhesion and movement, as well as, cell signaling. Dysregulated transport of these ions, occasionally caused by ion channel mutation, are associated with various "channelopathies".[2]

During the past two decades, synthetic systems capable of mimicking the functions[3] of natural ion transporters were focused extensively to understand the fundamental knowledge of ion transport mechanism. Subsequently, these synthetic ion transporters were applied as antibacterial, anticancer, and antiviral agents.[4] For example, cation selective cyclic peptides, reported by Ghadiri and coworkers, act preferentially on bacterial membranes and these peptide based tube can collapse transmembrane ion potentials and cause rapid cell death.[5] Recently, few small molecule cation transport systems with potential antitumor activities have been reported.[6] Compelling evidence is also available which indicates the regulatory role of $K^+$ and $Na^+$ during both the initial signaling and the execution phase of apoptosis.[7] Interestingly, the extracellular pH around the tumor microenvironment is typically very acidic while the intracellular pH is alkaline.[8] On the other hand, natural ion channel such as valinomycin is highly selective towards $K^+$ and consequently generates membrane potential. However, most of the synthetic transporters cannot generate membrane potential due to lack of selectivity. Therefore, synthetic molecules capable of perturbing both cation and anion homeostasis in this deviated pH range and having potential ability to generate membrane potential, can be applied to improve the combination treatment modality in addition to the conventional proapoptotic chemotherapy and radiotherapy.

Inversion of the ion transport selectivity as a result of pH change can be envisaged as an alternate approach for the transporter development. However, such approach has been less efficient till date because the inversion of selectivity is less pronounced within the pH range narrowly deviated from the physiological value. For example, the dimeric alamethicin analog with lysine at position 18 in the sequence (alm-K18) system was shown to form stable anion-selective channels in membranes at pH 7.0 while cation selectivity was prominent only at pH > 11.[9] Kobuke and co-workers have reported tetracyanoresorcin[4]arene derivative as a potential $K^+$ channel at higher pH due to deprotonation of hydroxyl groups on the resorcinarenes.[10] Later on, Gin and co-workers have reported a pH-sensitive aminocyclodextrin based ion channel, which shows faster anion and cation transport rate at higher pH.[11] Therefore, efficient protonation and deprotonation by marginally altering the pH from the physiological value can allow a synthetic system to inverse its selectivity towards either cation or anion. Herein, we report a class of bis(sulfonamides) as transmembrane ion carrier which displays $Cl^-$ selectivity at pH 5.5, while $K^+$ selectivity at pH 7.0.

Sulfonamides are an important class of compounds, which can act as receptors for anions,[12] as well as cations.[13] Therefore, the acidity (*i.e.* p$K_a$) of the N−H bond can be considered as one of the dictating factors behind its binding mode. An optimum p$K_a$ value can favor



| Compound | R | p$K_a$ of N-H | logP |
|---|---|---|---|
| **1a** | $-CH_3$ | 7.31 | 4.01 |
| **1b** | $-NO_2$ | 6.85 | 3.80 |
| **1c** | –Br | 7.14 | 5.38 |
| **1d** | $-OCH_3$ | 7.39 | 3.57 |
| **1e** | $-CF_3$ | 7.14 | 5.84 |

**Fig. 1** Structure, estimated p$K_a$ and logP values of designed bis(sulfonamides) **1a**-**1e**.

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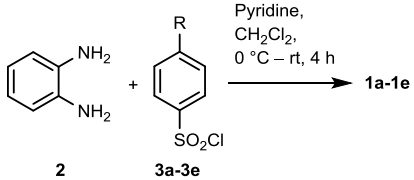
the neutral state of the sulfonamide at physiological pH, leading to facilitated anion binding. Further lowering of p$K_a$, based on structural change, can assist the deprotonation of N−H bond at physiological pH featuring cation binding. Earlier, we have reported bis(sulfonamides), formed on the *m*-xylylenediamine core, which displayed anionophoric behavior.[14] In the present study, we have introduced *o*-phenylenediamine as the core to design bis(sulfonamides) **1a-1e** (Fig. 1). Substituents at the *para*-position of aryl sulfonyl moieties were varied (R = -$CH_3$, -$NO_2$, -Br, -$OCH_3$ and -$CF_3$ for **1a-1e** respectively) to alter the p$K_a$ and logP values. Estimated p$K_{a1}$ = 6.85 − 7.39, using the calculator plugins of Marvin Sketch program,[15] indicated an increase in the possibility of deprotonation upon connecting electron withdrawing R groups and therefore, signifying better cation recognition. However, the change of R group also contributed in the predicted logP = 4.01, 3.80, 5.38, 3.57 and 5.83 for **1a-1e**, respectively (Fig. 1). According to the Lipinski's rule, a logP value close to 5 is crucial to attain improved membrane permeability, which directly translates into better ion transport activity.[16]

Syntheses of bis(sulfonamide) derivatives **1a-1e** were carried out from *o*-phenylenediamine **2** following the reported protocol by Proust *et. al*.[17] The reaction of **2** with aryl sulfonyl chlorides **3a-3e** in dichloromethane, and pyridine as base resulted in the formation of **1a-1e** in 78-96% yields (Scheme 1). All compounds were well characterized by $^1$H-NMR, $^{13}$C-NMR, HRMS, IR and melting point (see supporting information). Crystal structures of **1c** and **1e** were obtained from MeOH/$CHCl_3$ (1:1) solvent system, feature intramolecular N−H···O hydrogen bond between two arms around the central aromatic core (Fig. S8-S9).

Ion transport activities of **1a-1e** across large unilamellar vesicles (LUVs) composed of egg-yolk phosphatidylcholine (EYPC) with entrapped 8-hydroxypyrene-1,3,6-trisulfonate dye (HPTS, 1 mM) in HEPES buffer (pH = 7.0) were evaluated by applying a pH gradient ΔpH = 0.8 ($pH_{in}$ = 7.0 and $pH_{out}$ = 7.8) (Fig. S1).[18] Ion transport activity of **1a-1e** indicated compound **1e** as the most efficient ion transporter (Fig. 2A, S2). These studies also provided the transport activity order: **1d** < **1b** < **1a** < **1c** <**1e**, based on the $EC_{50}$ = 40, 43, 14, 211 and 10 μM for **1a-1e**, respectively (Fig. 2B, S3). Lowest activity of **1d** explained based on its lowest N−H bond acidity (p$K_{a1}$ = 7.39) as well membrane permeability (logP = 3.57). Although, the p$K_{a1}$ = 6.85 was estimated for **1b**, its lower activity compared to **1c** and **1e** was corroborated the lower logP = 3.80. Obtained data indicated that the compounds **1c** and **1e** having logP value higher than 5, displayed higher ion transport ability as compared to other bis(sulfonamides). The higher activity of **1e** as compared to **1c**, can be corroborated to higher lipophilicity which is due to the presence of two –$CF_3$ groups (as fluoride atom increases lipophilicity of molecule). Based on this outcome, the effect of either extravesicular cation or anion on the transport activity of **1e** across EYPC-LUVs with entrapped HPTS was determined by applying a similar pH




| Compound | R | Yield |
|---|---|---|
| **1a** | $CH_3$ | 78% |
| **1b** | $NO_2$ | 86% |
| **1c** | Br | 80% |
| **1d** | $OCH_3$ | 88% |
| **1e** | $CF_3$ | 96% |

**Scheme 1** Synthesis of bis(sulfonamides) **1a-1e**.

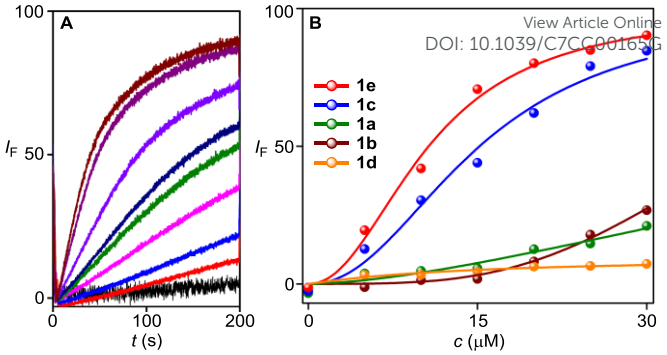



**Fig. 2** (**A**) Concentration dependent ion transport activity of **1e** with increasing concentration (0 – 15 μM) and (**B**) comparison of ion tansport activities of **1a-1e** presented with their normalized emission intensity $I_F$.

gradient in HEPES buffer.[19] Variation of NaX salts ($X^-$ = $F^-$, $Cl^-$, $Br^-$ and $I^-$) in the extravesicular buffer with iso-osmolar intravesicular NaCl provided minor differences (*i.e.* $Cl^- > F^- \approx Br^- \approx I^-$) in the ion transport activity of **1e** (Fig. 3A). However, the variation of extravesicular MCl ($M^+$ = $Li^+$, $Na^+$, $K^+$, $Rb^+$ and $Cs^+$) with iso-osmolar intravesicular NaCl resulted in the transport activity sequence: $K^+ > Rb^+ \approx Cs^+ > Na^+ > Li^+$ (Fig. 3B) for **1e** (2.5 μM). The observed data suggested that the ion transport of **1e** was largely influenced by the variation of cations in the extravesicular buffer. Therefore, preferred cation recognition by **1e**, possibly due to the deprotonation of bis(sulfonamide) N–H group under the assay conditions, was predicted. Similarly, **1c** also indicated a sharp influence of cations in transport activity (Fig. S4).

In the next stage, the carrier induced passive influx of cations were studied.[20] EYPC-LUVs were prepared by trapping NaCl (100 mM) and HPTS (1 mM) in HEPES buffer (pH = 7.0). The extravesicular salts in the same buffer were varied from NaCl to KCl. Without applying any initial pH gradient, the addition of **1e** ($c$ = 5 μM) resulted in the steady increase of HPTS fluorescence (Fig. 4A) when the extravesicular buffer contains NaCl. Further application of the pH gradient provided a sharper increase of the fluorescence. In the presence of extravesicular KCl, the addition of **1e** led to the sudden initial increase influorescence followed by a slow diminution with time (Fig. 4B). The faster increase in initial fluorescence clearly corroborates to a very fast influx of $K^+$ resulting in the generation of transmembrane potential due to the

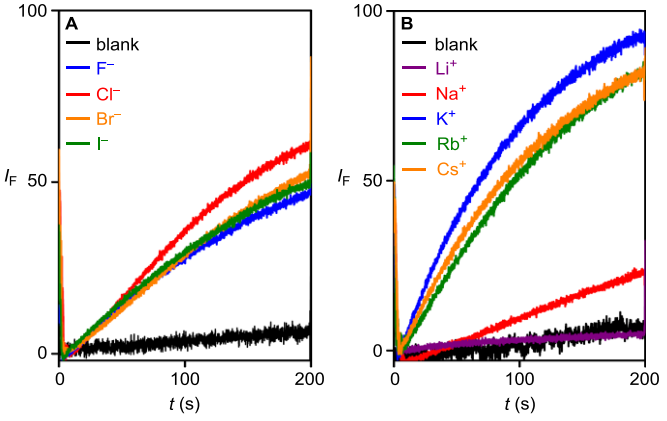



**Fig. 3** (**A**) Anion selectivity of **1e** (10 μM) and (**B**) cation selectivity of **1e** (2.5 μM) determined from HPTS assay.

accumulation of more positive charge inside, and negative charge outside the bilayer membrane. The creation of transmembrane potential further induces efflux of $H^+$ synergistically to restore the electrical balance. Subsequent decrease of fluorescence was associated with the slow $H^+$ influx to equilibrate pH gradient caused during $K^+/H^+$ antiport. Further addition of NaOH resulted in an abrupt increase in the internal HPTS fluorescence suggesting a rapid $H^+$ efflux for equilibrating the pH.

To obtain the direct proof of membrane polarization as a result of $K^+$ transport, safranin O (a membrane potential sensitive fluorescent dye) was used during ion transport studies.[20-21] EYPC-LUVs were prepared with filled KCl (100 mM) in HEPES buffer (pH = 7.0) and dispersed in isoosmolar NaCl buffer and safranin O (60 nM). $K^+$ efflux, upon addition of **1e** (5 μM), resulted in the rise of positive charge outside bilayer membrane which in turn generated transmembrane potential. The process led to a sharp increase in the safranin O fluorescence (at $\lambda_{em}$ = 581 nm with $\lambda_{ex}$ = 522 nm), confirming the generation of membrane potential due to selectivity towards $K^+$ (Fig. 4C). Fluorescence intensity was normalized by taking intensity at $t$ = 300 s as maximum point.

The aforestated outcome of cation selectivity of **1e** encouraged us to investigate the effects of pH on its ion selectivity. LUVs were prepared with entrapped HPTS dye. In the extravesicular MX salt, either anions ($X^-$ = $F^-$, $Cl^-$, $Br^-$ and $I^-$) or cations ($M^+$ = $Li^+$, $Na^+$, $K^+$, $Rb^+$ and $Cs^+$) were varied with iso-osmolar intravesicular NaCl. For each experiment, the intravesicular and extravesicular pHs were kept same (*i.e.* either 5.5 or 7.0 or 8.0). Cation selectivity (mostly towards $K^+$) was observed when compound **1e** (7.5 μM) was added to the cuvette containing LUVs at pH = 7.0 and 8.0 (Fig. 5A). However, no significant anion selectivity was observed at these pHs. When same experiments were carried out at pH = 5.5, significant anion selectivity (mostly towards $Cl^-$) was observed, and selectivity among cations became insignificant (Fig. 5B). All these results suggest that **1e** can bind to a cation at pH ≥ 7.0, and at acidic pH, *i.e.* pH = 5.5, the selectivity of the system gets reversed towards anions.

To establish further the pH dependent ion selectivity of **1e**, chloride efflux across bilayer lipid membranes were studied at different pHs by chloride selective electrodes (ISEs).[22] Chloride ion efflux by **1e** (20 μM) across EYPC-LUVs at different pH (*i.e.* intravesicular and extravesicular pH = 5.8, 7.0 and 8.0) with intravesicular NaCl (500 mM), and iso-osmolar extravesicular $NaNO_3$ indicated maximum activity at pH = 5.8 as compared to that observed at pH = 7.0 and 8.0 (Fig. 6A). These data reconfirms that

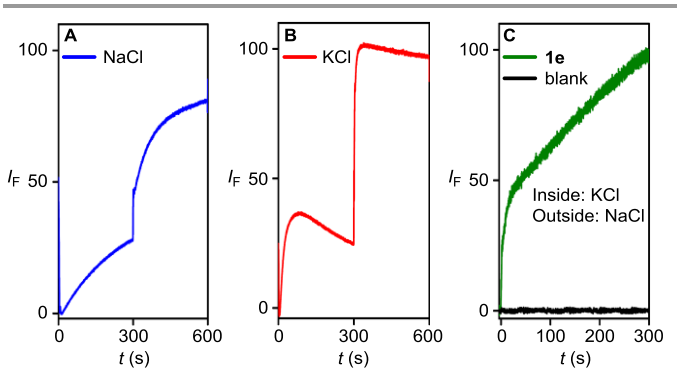



**Fig. 4** Normalized fluorescence intensity with time in extravesicular (**A**) NaCl buffer, (**B**) KCl buffer with compound **1e** (5 μM) and (**C**) fluorescence based membrane potential experiment with Safranin O dye.

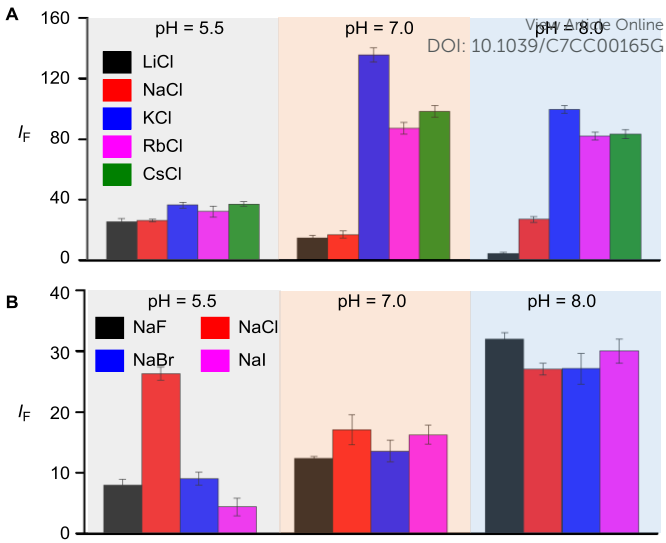



**Fig. 5** (**A**) Cation selectivity of **1e** determined from HPTS assay and (**B**) anion selectivity of **1e** determined at different pH without applying base pulse.

**1e** is a preferential cation transporter at high pH, *i.e.* in its deprotonated form, and an anion transporter at low pH, *i.e.* in its protonated form, establishing the pronounced selectivity switching effect by pH as the signal. Concentration dependent $Cl^-$transport activity profile of **1e** (Fig. 6B) at pH = 5.8 provided $EC_{50}$ =14 μM (Fig. S6) and initial rate ($I_R$) was found to be 5.27 × $10^{-3}$ $s^{-1}$ at $c$ = 15 μM (Fig. S7).

To attain theoretical insight about the geometry of **1e** with $K^+$ ion complex, initial structures were generated by using CONFLEX 7 program.[23] A [(**1e**)$_2$•$K^+$] complex was considered based on the Hill coefficient value obtained from EYPC-LUVs⊃HPTS assay. Initial geometries were predicted by using MMFF94s force field of the software and the conformation with highest Boltzmann population (85%) was selected for the geometry optimization study. The most populated structure was optimized by Gaussian 09 program package[24] using B3LYP functional and 6-311G (d,p) basis set[25] and most stable conformation of [(**1e**)$_2$•$K^+$] complex was obtained. The optimized structure shows binding with $K^+$ ion with two molecules of **1e** through the electrostatic bond between deprotonated N–H group of bis(sulfonamide) **1e** and one molecule of $K^+$ ion (Fig. 7) validating the obtained cation selectivity and mode of binding.

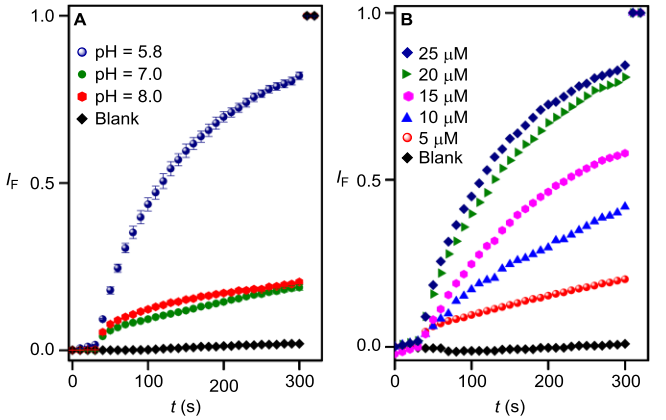



**Fig. 6** (**A**) Chloride ion selectivity of **1e** (20 μM) at different pHs and (**B**) chloride Ion efflux of **1e** with increasing concentration (0 – 25 μM) determined by using ISE.

**Fig. 7** Geometry optimized structure of [(**1e**)$_2$•K$^+$]. Color codes: green = potassium, blue = nitrogen, yellow = sulfur, maroon = oxygen, blackish grey = carbon, cyan = fluorine.

In conclusion, we demonstrated the design and syntheses of bis(sulfonamides) bearing five different groups to tune their N−H acidity and lipophilicity attributes. Ion transport activity and selectivity were extensively studied by several fluorescence based assays with EYPC liposomes. They displayed strong cation selectivity as compared to anion selectivity due to binding of the cation with deprotonated derivative at physiological pH. This cation selectivity was found to be dependent on pH and it switched to anions electivity at acidic pH. This ion selectivity flipping behavior could be realized within very narrow pH range. This observation was further reiterated by monitoring chloride efflux at different pH by chloride selective electrode. Passive transport of $K^+$ and generation of transmembrane potential for selective transport of $K^+$ was also demonstrated by monitoring fluorescence of Safranin O. The mode of binding with $K^+$ was also showed by theoretical calculations.

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