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ORIGINAL ARTICLES

Synthetic Anionophores for Basic Anions as "Presumably, OH^-/Cl^- Antiporters": From the Synthetic Ion Channels to Multi-ion Hopping, Anti-Hofmeister Selectivity, and Strong Positive AMFE

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Abstract We describe application of theory and kinetic modeling to study transport of basic anions by the small synthetic molecules. The findings should equip researchers in the particular field with a tool necessary to address an essential question: whether a given anion transporter facilitates permeation of F^-, CH_3COO^-, N_3^-, and SCN^- across biological membrane or it does not. The basic anions undergo hydrolysis and conjugate acids (HAnion) are permeant species. However, because methods to quantitatively account for HAnion transport do not exist, traditionally, the phenomenon is also treated as non-existing. When the relative activities and selectivity of the synthetic anionophores are evaluated, basic and non-basic anions are regarded in the same exact way. Here, we show that HAnion and H^+/OH^- transport proceed on the same time scale as the anion exchange, nevertheless, comprehensive kinetic study could provide solution to the problems at hands, such as selective transport of HCO_3^- or F^- anions. We also use theory and modeling to study other questions of particular concern: transport of OH^- and H^+ ions, facilitated by the small synthetic anionophore, origin of modified anti-Hofmeister selectivity, multi-ion hopping, and anomalous mole-fraction effect in the synthetic ion channels. We do not need to model kinetics in a synthetic channel with multiple ion binding sites. Instead, we "test" the most simple anionophore, a lipophilic electroneutral carrier with Hofmeister-like selectivity, in the classical assays as "presumably, Cl^-/OH^- antiporter." The implications of findings to the particular field and beyond are discussed.

Keywords Proton and hydroxide permeability · Anion exchange · Anionophore · Anomalous Mole-Fraction Effect (AMFE) · Synthetic ion channels · Basic anions

Introduction

Number of small molecules, natural and synthetic, able to transport inorganic anions (including basic F^-, CH_3COO^-, HCO_3^-, and SCN^-) across biological membrane, is rapidly growing (Davis et al. 2007, 2009; Gale et al. 2013; Jentzsch et al. 2013; Valkenier and Davis 2013). The classical techniques rely on pH-sensitive dye HPTS (1-hydroxypyrene-3,6,8-trisulfonate) incorporated in lipid spherules and "presumable, OH^-/Cl^- antiport" reports on the anion selectivity and mechanism (Matile and Sakai 2007, 2012; Sakai and Matile 2006).

Back in 2009 we described new HPTS assays. Accordingly, the intravesicular pH reports on the anion exchange, which is electrogenic phenomenon. In this case, the anion permeabilities, absolute or relative, are parameters of interest and those can be easily calculated from the data (Berezin and Davis 2009). More recently, we used theory and modeling to illustrate that the old method is indeed delusive (i.e., the anion transporters with Hofmeister-like selectivity will exhibit apparent anti-Hofmeister selectivity in the assays developed by Matile and colleagues, and vice versa) (Berezin 2013). In our studies, however, we focused on the non-basic anions. What one may expect to see in the case of basic anion in the classical supramolecular assays, and whether HPTS-based method can be used to obtain permeability data for basic anions—have not yet been discussed.

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Table 1 Physical properties of the selected monovalent anions and conjugate acids

Anion	$P_{Anion} \times 10^9$ (cm s^{-1})	ΔG_{Hydr} (kJ/mol)[c]	pK_a HAnion	P_{HAnion} (cm s^{-1})
F^-	0.83[a]	−404	3.2	3.1×10^{-4}[d]
SCN^-	41[a]	−275	1.0	2.6[d]
$C_2H_3O_2^-$	1.3[a]	−389	4.8	5.0×10^{-3}[d]
N_3^-	7.6[a]	−331	4.7	6.5×10^{-2}[a]
Cl^-	2.2[b]	−363	−8	2.9[d], 3×10^{-3}[e]
Br^-	7.3[b]	−328		
NO_3^-	19[b]	−311	−1.3	9.2×10^{-4}[d], 4×10^{-7}[e]
I^-	60[b]	−278		
ClO_4^-	116[b]	−228		

[a] Calculated using theory and literature data (see details in the SI)

[b] Literature data (Berezin and Davis 2009)

[c] Literature data, extrapolated from the electrode measurements (Smith et al. 1999)

[d] Literature data (Walter and Gutknecht 1986)

[e] Literature data (Nozaki and Tanford 1981)

Recently, Cl^--selective electrode was used to access transport of strongly basic bicarbonate, and Hofmeister-like selectivity $SO_4^{2-} < HCO_3^- < NO_3^-$ was established for many synthetic anionophores (Busschaert et al. 2010, 2011; Davis et al. 2009; Gale et al. 2010; Hussain et al. 2011). In this case, presumable Cl^-/HCO_3^- antiport is a process in focus. Accumulation of bicarbonate inside lipid spherules or its release can also be directly detected using ^{13}C-NMR and paramagnetic Mn^{2+} ions proving that liposomes indeed remain intact upon exposure to these membrane-active agents (Davis et al. 2009). Though originally devised to study small synthetic molecules, this technique can be used to study HCO_3^- transport proteins, co-transporters or exchangers, reconstituted in lipid spherules (Alvares-Leefmans and Delpire 2009).

The growing interest toward bicarbonate is due to potential therapeutic (or anticancer) properties of the agents which are able to restore defective HCO_3^- (and Cl^-) transport in the living cell (or to disrupt the ion balance inside the tumor) (Davis and Sheppard 2012; Gale 2011). Thus, HCO_3^- selective receptors can be identified (Hiscock et al. 2009), and the long-standing goal is to find bicarbonate-selective transporters (Gale 2012). Finally, interesting to note that some of the bicarbonate transporters, reported so far, like the natural product prodigiosin (Davis et al. 2009), are preorganized for the selective recognition of HCO_3^- (contain H-bonds rationally placed and spaced), while the others, like calix[4]pyrroles (Gale et al. 2010), are not.

Apart from bicarbonate anion, synthetic compounds that selectively transport F^-, SCN^-, and CH_3COO^- over non-basic ClO_4^- and Cl^- across liposomal bilayer are the most intriguing. Examples include but not limited to naphthalenediimide (NDI)-based pi-slides, NDI monomers (Davis 2010; Dawson et al. 2010; Gorteau et al. 2006, 2007; Mareda and Matile 2009) and recently reported perfluorinated alkyl halides (Jentzsch et al. 2012). Importantly, all these molecules are thought to implement "exotic" ways to bind ions inside biomembrane (i.e., halogen bonds, anion–π, and/or anion–macrodipole interactions). For instance, the self-assembling oligourea/amide macrocycle relies on anion–macrodipole interactions and selectively brings SCN^- across lipid bilayer (Hennig et al. 2009); in turn, the anion–pi slides are F^--selective. Although all the kinetic data are questionable (as obtained using classical HPTS techniques), traditionally, the findings have also been supported by relevant non-kinetic studies such as ESI MS analysis and high level DFT calculations [electrospray ionization mass spectrometry (ESI MS), density functional theory (DFT)]. Moreover, recent reports by Saha and colleagues demonstrated that "exotic" π–acidic surfaces can indeed be particularly selective for F^- anion in the mixed aqueous solutions. Accordingly, two NDI units, rationally placed and spaced by means of a folded linker, lead to a highly sensitive receptor which selectively binds F^- over other halides as demonstrated by ITC measurements ($K_a(F^-) = 10^9$ M^{-1} versus $K_a(Cl^-) = 10^3$ M^{-1}, where K_a the association constant, ITC the isothermal titration calorimetry) (Guha et al. 2011; Guha and Saha 2010; Saha and Guha 2013).

Simply because in the supramolecular field transport of F^- has been investigated using old HPTS technique only, question—whether synthetic anion receptors can employ "exotic" or classical interactions to translocate this anion across biomembrane—remains wide open. Two mechanisms discussed in literature—a (multi)anion hopping (along π–acidic surfaces) as F^--selective passage way (Davis 2010; Dawson et al. 2010; Gorteau et al. 2006, 2007; Mareda and Matile 2009) and a relatively simple F^--selective carrier—both look attractive. Possible advantages that anion–pi interactions might encode over classical H-bonds, in respect to development of potential prodrugs for treatment of cystic fibrosis, have been recently mentioned elsewhere (Jentzsch et al. 2012). Hence, recent advances in "exotic" recognition of inorganic anions in water and mixed aqueous solutions, which includes halogen bonds, CH-anion contacts along with anion–pi, should also be highlighted (Arranz-Mascaros et al. 2013; Berryman et al. 2007; Chifotides and Dunbar 2013; Frontera et al. 2011; Gil-Ramírez et al. 2008; Guha et al. 2012; Wang and Wang 2013).

Our own interest toward F^- in particular is guided by the recent discovery of mechanism that regulates resistance of bacterial cell toward this toxic ion (Baker et al. 2012;

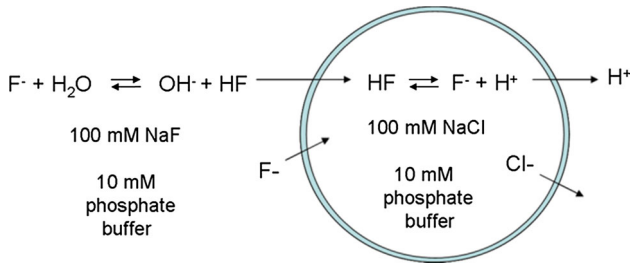

Fig. 1 F^-/Cl^- exchange. Two mechanisms of F^- translocation across biomembrane modified with synthetic anionophore

Stockbridge et al. 2012). Thus, CLC^F anion transporting protein initiates F^-/H^+ exchange (1:1 stoichiometry) and exhibits high selectivity for F^- over Cl^- ion. A small synthetic molecule, which is a F^--selective transporter, could become a valuable tool for biologists.

In contrast to "hard" alkali metal cations, the isoelectronic halides possess comparable radii and relatively "soft" (relatively polarizable and deformable) electronic shell. These features make it difficult to differentiate between these spherical anions using "key-lock" principle only. We note that the particular strategy worked remarkably well when researches' goal was to differentiate between spherical cations of the alkali metals, properties of which are quite opposite. To their advantage, altogether inorganic anions come in variety of shapes and topology of the electronic structure, offering a particularly interesting target for selective and reversible recognition. One should consider not only classical supramolecular hosts but also transition metals and metalloids, which recognize electronic shell by forming *selective but reversible* bonds. For instance, simple triorganotin compounds have long been known to mediate exchange of Cl^-, Br^-, SCN^-, and I^- for OH^- across lipid bilayer but selectivity and mechanism of this process are little understood (Selwin 1976). In turn, aluminum(III) complexes of porphyrins (Badr and Meyerhoff 2005) could be used to develop F^--selective carriers that work in biomembrane [Perfluorinated porphyrins might be suggested, as fluorinated anionophores are known to perform better inside the bilayer than non-fluorinated analogs (Bahmanjah et al. 2012; Gale et al. 2010).] A scientist should readily conclude that new field offers an outstanding opportunity for a chemist interested in ion recognition. Instead of focusing on high *sensitivity* of recognition phenomena one can seek to combine *reversibility* with high degree of selectivity in a provisional transporter.

To guide research efforts, one should be able to relate the structure to the activity and selectivity of the synthetic compound. And there is an important difference between anions of the strong acids and basic anions, such as F^-, as the conjugate acids are present in solution, and these are the permeant species (Fig. 1).

The same is valid for other basic anions to be discussed but not bicarbonate (when corresponding salts replace extravesicular F^- depicted in Fig. 1). H_2CO_3 is particularly weak acid, $pK_{a1}(H_2CO_3) = 6.4$, which exists in two forms, H_2CO_3 and CO_2. Both species are permeant. In turn, CO_2 in solution is in "equilibrium" with atmospheric CO_2. This system appears as more complex, and its analysis goes beyond the scope of this study.

In the absence of the anionophore, one may expect to see decrease in the intravesicular pH (Fig. 1), which was observed when basic anions were evaluated using classical HPTS assays (Gorteau et al. 2006, 2007). Moreover, back in 2009 we reported "cute trick," NaN_3-based assay to study the selectivity of the anion translocation, where sum of HN_3 efflux and H^+ influx produced flux of N_3^- anions (Berezin and Davis 2009). 5 years past but yet there is no understanding of these phenomena, which explains why "weak acid mechanism" simply does not exist in the case of novel synthetic bicarbonate transporters (Busschaert et al. 2010, 2011; Davis et al. 2009; Gale et al. 2010; Hussain et al. 2011) and why this NaN_3-based assay is also non-existing.

Herein, we describe transport of basic anions theoretically using the GHK-based model [GHK—Goldman–Hodgkin–Katz theory (Hille 2001)]. Because "presumable, OH^-/Cl^- antiport" remains a convenient method to study transport of anions of the weak acids, meanwhile H^+ and OH^- translocations by small synthetic molecules are not understood, we address all these questions as well.

Working with a single permeation barrier, GHK alone is insufficient. The state of art in the field requires one to describe interactions within the bilayer. First, since a single barrier does not adequately describe "valinomycin for anions," and two-barrier one binding site model would be the most appropriate (SI). Second, but most importantly, since supramolecular chemistry has traditionally been focused on the synthetic channels and not the low-turnover transporters. Both cation and anion-selective synthetic ion channels have been made decades ago. [For reviews see, for instance (Fyles 2007; Gokel 2000; Gokel and Carasel 2007; Gokel and Daschbach 2008; Gokel and Mukhopadhyay 2001; Otis et al. 2013). For some recent examples, see (Leevy et al. 2002; Li et al. 2009)]. For example, the first functional synthetic analogs of Kcsa K^+ incorporated crown ethers and were reported just months apart by Gokel and Fyles (Gokel and Carasel 2007).

At present, the most widely used definition of synthetic ion channel relies on a specific result of the patch clamp or voltage clamp experiments—characteristic jump in conductance, or opening (and closing) events. (For examples of the synthetic ion channels which do not fall under this definition see Madhavan et al. 2005; Satake et al. 2008). Lately, some researchers started to define it as a channel

only if the membrane-active compound is long enough to span whole length of a bilayer; or (and) added into solution at the lowest possible concentrations. In this case, one can speculate that this jump in conductance indeed would always arise as a result of activity of exactly the same single molecule or assembly in the biomembrane, and hence, analyze its properties accordingly. (See, for instance Iqbal et al. 2007; Langecker et al. 2012; Yamnitz et al. 2010). Meanwhile, other researchers reported lack of consistent selective behavior (or reproducible selectivity) in the case of selective artificial ion channels derived using classical crown ethers as a repeating unit (Tsikolia et al. 2009).

In turn, the relative simplicity of the experiments with liposomes has always been particularly intriguing. For example, using parameter identified as % of transport at 400 s (or the initial rates calculated accordingly) Voyer and colleagues established that 6 Å is an optimum distance for Na^+ ion to travel between the two crown ether residues inside reconstituted bilayer, while up to 11 Å is well tolerated. The authors clarify that these results compare well with the findings reported for other natural and artificial ion channels. For instance, a maximum distance of 14 Å was found for Na^+ in crown ether-based hydraphile channels discovered by Gokel et al. Likewise, the open form of gramicidin A possesses two sites separated by 11.6 Å (Otis et al. 2011, 2013).

Presently, there is no doubt that phospholipid spherules are indispensible tool to characterize the artificial ion channels. But there is no agreement on nature of the observed phenomena. In contrast to the classical views popularized by Matile and colleagues, some other researchers proposed that the difference in kinetic response arises due to the difference in "activation" or "membrane insertion" dynamics of the synthetic ion channels.

The key points may be identified as following: (1) the dependence of activity of artificial ion channels on nature of electrolytes in the liposomal suspension is well documented but not understood; (2) in the case of only a single "open-close event" it will take just a few milliseconds (not minutes) to equilibrate the intravesicular ionic content of a single liposome with the extravesicular medium in a well-stirred solution (Miller 1984); (3) contrary to popular belief, "open(-close)" events will not preclude synthetic compound from functioning as a low-turnover anion transporter in liposomes or in a living cell (Harrell et al. 2010).

Among all the synthetic ion channels, unimoleculuar G-quadruplex that transports Na^+ in vesicles, yet stands apart (Arnaud 2005; Forman et al. 2000; Kaucher and Davis 2006; Kaucher et al. 2006). Created through a spontaneous assembly of 16 pieces of lipophilic guanosine derivative around a fine row of three K^+ ions, the interior is strikingly reminiscent of Kcsa K^+ selectivity filter where cations, in the adjacent binding sites, strongly interact with each other. Likewise, in each of the binding sites, K^+ ion is located at the center of cage formed by 8 oxygen atoms from the carbonyl groups of guanosine, sharing all or a half of its coordination sphere. Being chemists, we expect the cations to move along in a single file but yet have nothing to say about rate this process (or even whether it indeed takes place). The lack of activity in the voltage clamp setup would not disprove its channel-like property. In turn, the presence thereof will not make it an ion channel. Ions might move across, through lipophilic G-quadruplex with rates similar to the diffusion in the bulk solution or not even close to it. In theory, if for some ions the process is slow enough it could be monitored using lipid spherules.

In theory, the oversimplified kinetic model used herein, which implies no binding sites inside the bilayer, can still be useful to study relevant complex phenomena. For instance, apparent values are sufficient to evidence anomalous mole-fraction effect (AMFE) in the natural systems. In turn, this phenomenon supports a (multi)ion hopping, a channel mechanism where ions interact with each other inside a transmembrane pore (SI) (Hille 2001).

When two ions simultaneously traverse a pore (which is just few Å long) their movement may not be independent. For instance, when Ca^{2+}-selective ion channels are reconstituted into planar bilayer, there is a non-monotonic variation in macroscopic current with concentration of this permeant ion. This phenomenon is known as AMFE. In the absence of Ca^{2+}, the channel is merely selective and allows passage of any monovalent ion, which is small enough to squeeze through. In turn, at the low concentrations of this ion, the electrical current drops because Ca^{2+} efficiently occupies one of the two adjacent binding sites blocking a "single-filing" pore. However, when concentration of the permeant ion increases above a certain value the macroscopic current rises again because two Ca^{2+} ions in the adjacent binding sites efficiently destabilize each other making a "single-filing" pore to conduct again (Miller 1999).

Remarkably, small molecules also exhibit AMFE (Dawson et al. 2010; Gorteau et al. 2006, 2007; Hennig et al. 2009; Izzo et al. 2008; Jentzsch et al. 2012; Mareda and Matile 2009). But in supramolecular chemistry, AMFE is measured using lipid spherules. In this case, the parameter of interest is not the electrical current but "presumably, Cl^-/OH^- exchange," obtained from the changes in the intravesicular pH followed after the base pulse. The anion–π slides (Gorteau et al. 2006) are, probably, the most famous example where Matile and colleagues stated: "According to this classical test, the underadditivity found for Cl^-/I^- mixtures suggests that occupation of one single site with the better binding Cl^- is insufficient for fast Cl^- transport. Occupation of multiple sites along the π slide is thus required for the high activity found with pure Cl," citing the most relevant work by Hille

and Schwarz (1978), Miller (1999), and Tabcharani et al. (1993).

It strikes that at present the field still remains in its infancy state as the novel assays as well as development of physical picture behind it and behind the classical transport data "have not been understood." We hope our findings will help young researchers who work on relevant problems but are not in position to question existing paradigms.

Materials and Methods: Theoretical Modeling

We have recently described in great details GHK-based kinetic models of cationic and electroneutral anion transporters. Using flowchart-based description and Berkeley Madonna software, we have reproduced the data in the classical assays and demonstrated advantages of using theory to understand complex supramolecular phenomena such as H^+Anion^- co-transport and anti-Hofmeister selectivity (Berezin 2013). Here, as far as basic anions are of concern, all we have to do is to include the flux of weak acid (HAnion) into the original flowcharts. In addition, in order to model the kinetic data, we also need to define permeabilities, P_{Anion^-}, m s^{-1} for basic anions and corresponding conjugate acids.

If synthetic compound exhibits lyotropic selectivity than anion permeabilities follow an order: $P_{ClO_4^-} > P_{I^-} > P_{SCN^-} > P_{NO_3^-} > P_{N_3^-} > P_{Br^-} > P_{Cl^-} > P_{CH_3COO^-} > P_{F^-}$, which implies that anion which is more readily "dehydrated" will be transported faster. In other words, the energy of transfer from the aqueous phase is key contributor to the height of the kinetic barrier. Therefore, we can use the values of anion permeabilities reported for TREN-based bis-catechol (Berezin and Davis 2009) and literature values of the hydration energies, ΔG_{Hydr}, for basic and non-basic anions, in order to assign some arbitrary P_{Anion^-}, m s^{-1} for N_3^-, CH_3COO^-, F^- and SCN^-, which have not been measured experimentally. The data obtained by the approximation along with the experimental values are shown in Table 1. Presumably, the TREN-based bis-catechol would transport these basic anions with these exact rates.

In turn, flux of the weak acid will be determined by two parameters, the acidity constant, pK_a (HAnion) and permeability of the acid, P_{HAnion}, m s^{-1}. The former is known while latter is an arguable parameter. Weak acids are electrolytes and P_{HAnion}, m s^{-1}, found in literature for the same chemical compound, differ orders of magnitude (Table 1). Insufficiencies of both the experimental approach and theoretical treatment are thought to account for this discrepancy. To measure P_{HAnion}, m s^{-1} experimentally one must define P_{H^+}, m s^{-1} as well. In turn, proton permeability is dramatically influenced by the way the bilayer, spherical or planar, is prepared. The value of P_{H^+}, m s^{-1} rises orders of magnitude due to the presence of impurities and due to oxidation of phospholipids, which happens upon interaction with atmospheric oxygen. In turn, "unstirred layer" creates an additional kinetic barrier, which may or may not be taken into account when P_{HAnion}, m s^{-1} are calculated. Yet, to the advantage of an experimentalist: (1) permeability of the weak acid can be easily determined experimentally; (2) even if these parameters are obtained using different methods, the relative values are often the same.

The "unstirred layer" is a noteworthy relevant phenomenon. Even in the most vigorously stirred solution, the liposomes are surrounded by layer of the unmixed water about 1000 nm thick, where transport is limited by diffusion. For an inorganic ion, which permeability is low, this effect is negligible. But in the case of a relatively permeant specie, such as electroneutral weak acid, the time it spends traveling through this unstirred water and time it needs to cross 30 Å lipid bilayer are comparable. Therefore, the apparent speed at which for instance, HF molecule crosses biomembrane is a result of combination of the two effects (SI).

Here, we use P_{HAnion}, m s^{-1} reported by Walter and Gutknecht (1986) (Table 1). [We note that some researchers suggested that these particular experimental values may be too high (Nozaki and Tanford 1981).] An alternative way to obtain the parameters of interest would be to use octanol–water partition coefficients, experimental or predicted. In this case, P_{HAnion}, m s^{-1} can be calculated using either a simple relation (Missner and Pohl 2009) or a complex theory (Bemporad et al. 2004).

As all the key parameters have been defined, we may construct the kinetic model. We do it in a step-wise manner, starting not with the anion exchange but with the flux of weak acid, in the absence thereof.

We describe a classical experiment where small aliquot (40 µl) of liposomal suspension (100 mM $Na^+Cl^-_{IN}$ and 100 mM $Na^+Cl^-_{OUT}$ in 10 mM phosphate buffer) is dissolved into 2 ml isosmotic solution of $Na^+Anion^-_2$ in 10 mM phosphate buffer. At the neutral or basic pH, the influx of weak acid into liposomes is described by a set of Eqs. (1)–(6) ($HAnion_2$ = HF is an example):

$$pH_{IN}(t) = 7.2 + \log((0.01 - [H_2PO_4^-]_{IN}(t))/[H_2PO_4^-]_{IN}(t)), \quad (1)$$

$$pH_{OUT}(t) = 7.2 + \log\left(\left(0.01 - [H_2PO_4^-]_{OUT}(t)\right)/[H_2PO_4^-]_{OUT}(t)\right), \quad (2)$$

$$[HF]_{IN}(t) = [F^-]_{IN}(t) \times [H^+]_{IN}(t) \times K_a(HF), \quad (3)$$

$$[HF]_{OUT}(t) = [F^-]_{OUT}(t) \times [H^+]_{OUT}(t) \times K_a(HF), \quad (4)$$

$$J = -d[F^-]_{IN}/dt \times V_{IN} = d[F^-]_{OUT}/dt \times V_{OUT} = P_{HF} SN\left([HF]_{IN}(t) - [HF]_{OUT}(t)\right), \quad (5)$$

$$J = -V_{IN} \times d\left[H_2PO_4^-\right]_{IN}/dt = V_{OUT} \times d\left[H_2PO_4^-\right]_{OUT}/dt = P_{HF} S\, N\left([HF]_{IN}(t) - [HF]_{OUT}(t)\right), \quad (6)$$

where V_{IN} and V_{OUT} are the volumes of the intra- and extra-vesicular solutions, N the number of liposomes, and S the surface area of liposome. Numerical values for all these parameters have been determined and comprehensively discussed in our recently published study (Berezin 2013). The origin of Eqs. (1)–(2) has also been discussed, while Eqs. (3)–(4) are straightforward. Equations (5)–(6) originate from definition of diffusion flux (plus the law of conservation of mass) and strictly speaking, are not precise but valid assumptions at pH ≥7. Because $HAnion_2$ is a weak acid, not all of it dissociates, yet concentration of $HAnion_2$ in solution is orders of magnitude below concentration of $Anion_2^-$ and so negligibly small. The problem is largely analogous to the titration of phosphate buffer using strong versus weak acid: as long as pH is far above the pK_a of the weak acids there is no difference between these two systems. However, permeability of $HAnion_2$ is many orders of magnitude higher than that of $Anion_2^-$ and so flux of $HAnion_2$ may (or may not) proceed on a relevant time scale.

Kinetic model described by Eqs. (1)–(6) can be visualized as Flowchart A in Fig. 2. We may reiterate that spheres and rectangles represent intra- and extra-vesicular solution, accordingly, thick arrows—flows, J, mol s^{-1}, while thin arrows—functional dependences.

If protons (or other ions) permeate across the bilayer on relevant time scale, transport phenomenon becomes electrogenic. How theory defines dependence of the flux of charged species on the electrical potential, E_m has already been comprehensively discussed (see also SI). In turn, E_m is explicitly related to the charge at the bilayer surface, which is expressed as a sum of number of cations that left the vesicles and numbers of anions that crossed the bilayer to get inside (and vice versa).

Since transport of one H^+ or one OH^- in the opposite direction leads to equivalent results, we could have operated with H^+ only, varying P_{H^+} in a certain range as we have done in our recent study (Berezin 2013). In this case, P_{H^+}, m s^{-1} is related not only to the protons but also to the net flux of OH^- and H^+ ions. Yet, here we have chosen to consider permeation of both ions, OH^- and H^+ separately. Thought this approach slightly complicates the description, it is needed in the field, where traditionally, H^+ and OH^- transports (and binding) have been differentiated from one another. Moreover, it should certainly carry advantages in a future.

The Flowcharts B and A in Fig. 2 are largely the same. The only difference is that fluxes of three different species, H^+, OH^-, and HAnion cause the pH change. Importantly, the value of the membrane potential, E_m is derived using changes in the intravesicular concentration of not all $[H_2PO_4^-]_{IN}$ but only tiny fraction of it, which is generated due to passage of H^+ and OH^- across the bilayer, depicted as inner circle. In other words, E_m is a sum of number of H^+ ions that left the vesicles and number of OH^- that crossed it to get inside.

Interesting to note that when charge and mass transport in system described by Flowchart B in Fig. 2 reach the equilibrium state, the transmembrane potential is determined by the GHK voltage equation:

$$E_m = \frac{RT}{F} \ln\left(\frac{P_{H^+}[H^+]_{OUT} + P_{OH^-}[OH^-]_{IN}}{P_{H^+}[H^+]_{IN} + P_{OH^-}[OH^-]_{OUT}}\right). \quad (7)$$

Contrastingly, when system depicted in Flowchart C in Fig. 2 reaches the equilibrium, the anion exchange is complete and $E_m = 0$. To describe dependence of ion fluxes on value of transmembrane electrical potential we use the GHK flux equation (SI). The modeling of anion exchange is straightforward and analogous to our recently published study so as not to be described in details. We should only mention two separate fractions of $Anion_2^-$ that permeate across the bilayer by means of electrogenic and electroneutral pathways as Flowcharts B and C in Fig. 2 are largely analogous. Finally, the kinetic models in Fig. 2 are schematic presentations, complete mathematical models for the solutions depicted in the "Results and Discussion" section can be found in the SI.

The only remaining question is: What are the relevant values of P_{OH^-} and P_{H^+}, and which should be used for modeling? Specific mechanisms have been proposed to account for sometimes anomalously high net H^+/OH^- permeability in pure lipid bilayer. A proton hopping along the water wire (Grotthuss mechanism) and weak acid impurities (e.g., free fatty acids) have been discussed and reported values of P_{H+} are 10^{-6}–10^{-11} m s^{-1} (Deamer and Nichols 1983; Gutknecht and Walter 1981). Therefore, here, considering the lowest limit and taking into account protonophoretic activity of some anion transporters we simply vary P_{H^+} in a range 10^{-3}–10^{-11} m s^{-1}. ($P_{H^+} = 10^{-3}$ m s^{-1} is close to the diffusion limit, where rate is determined by diffusion through the "unstirred layer").

In turn, reported value of P_{OH^-} equals 10^{-9}–4×10^{-11} m s^{-1} (Gutknecht and Walter 1981; Nozaki and Tanford 1981), though the mechanism of hydroxide translocation is not understood. On the other side, OH^- permeation in the absence of anionophore, across unmodified lipid bilayer, is largely irrelevant to the problems at hand. Because hydration energies for Cl^- and OH^- are

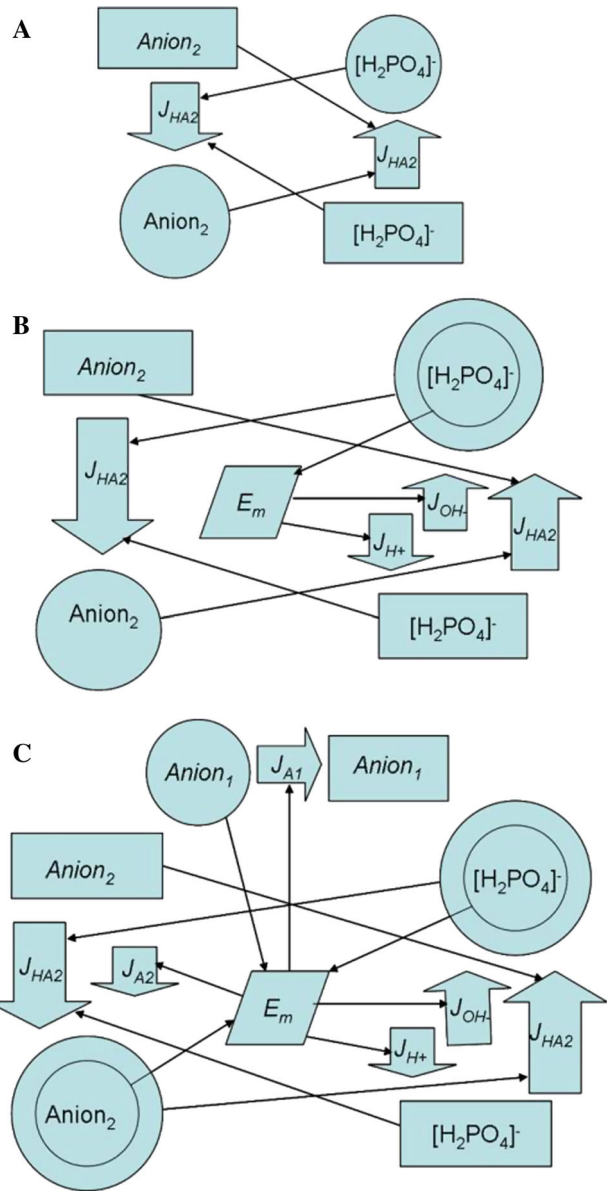

Fig. 2 General structure of the flowcharts. $HAnion_1$ is a strong acid and $HAnion_2$ is a weak acid. Permeant species: **a** $HAnion_2$; **b** $HAnion_2$, H^+, and OH^-; **c** $Anion_1^-$, $Anion_2^-$, $HAnion_2$, H^+, and OH^-

similar and so as the permeabilities, we may have assigned $P_{OH^-} = 2.2 \times 10^{-11}$ m s^{-1} (Table 1). However, since aforementioned lower limit for hydroxide permeability across unmodified lipid bilayer is yet higher than P_{Cl^-}, m s^{-1} we use $P_{OH^-} = 4 \times 10^{-11}$ m s^{-1} instead.

Therefore, if synthetic compound with Hofmeister-like selectivity transports Cl^- with the same rate as TREN-based bis-catechol, number of OH^- anions translocated across by this molecule will be just a small fraction of those that permeates across on its own. In turn, if in the same assay Cl^- transport proceeds with the same rate as ClO_4^- and so $P_{OH^-} \approx 10^{-9}$ m s^{-1} (Table 1) (or we could have increased concentration of TREN-based bis-catechol accordingly), the rate of OH^- translocation by this receptor will be the same or above the rate of unassisted passage. Nevertheless, because aforementioned upper limit, $P_{H^+} = 10^{-6}$ s^{-1}, is higher orders of magnitude, the best chances are the experimentally observed changes in pH in the classical assays will be due to unassisted passage of H^+ across unmodified lipid bilayer.

It is relevant to note that for our analysis we used the literature data on OH^-/H^+ permeability of the reconstituted bilayer, and phosphatidycoline and cholesterol are its key components. In the case of a living cell P_{OH^-/H^+}, m s^{-1} should depend on its type and function. In turn, within a cell P_{OH^-/H^+} of the energy-transducing organells, chloroplasts and mitochondrial are expected to differ from P_{OH^-/H^+} of a cytoplasmic membrane.

Finally, in the recent study we pointed out the possibility of the anionophore-mediated transport of $H_2PO_4^-$ (Berezin 2013). (In the more basic medium, the dihydrophosphate breaks apart to produce H^+ and HPO_4^- ions.) Likewise, here we have chosen to exclude this process which only produces unnecessary complexity. The concentration of $H_2PO_4^-$ is relatively small in the intra- and extra-vesicular solution. The total concentrations of phosphate anions in the two compartments are expected to vary in the presence of an anionophore during the anion exchange (but the two are exactly the same at the end of the process). These changes may affect the parameters of interest. However, the absolute values are irrelevant to the problem at hand. The aforementioned process should be revisited, for instance, in analysis of a phosphate-selective anionophore or in any other case where the precision matters. In order to understand the phenomenon and its effect, a researcher can always employ advantages of the experimental approach by changing the nature of the buffer and its concentration.

Results and Discussion

Transport of the Weak Acids and H^+/OH^- ions

The modeling results for the two systems described by the flowcharts A and B in Fig. 2 are depicted in Fig. 3. The data illustrate that even at basic pH, transport of the weak acid $HAnion_2$ proceeds on the same time scale as the anion exchange mediated by a synthetic transporter. May the real values of $P_{HAnion2}$ be smaller, even orders of magnitude, the process would still be relevant phenomenon around neutral pH. In turn, transport of H^+ and OH^- ions further shapes the pH on time dependences. Importantly, there is no changes in pH for NO_3^-—a non-basic anion with $pK_a = -1.3$.

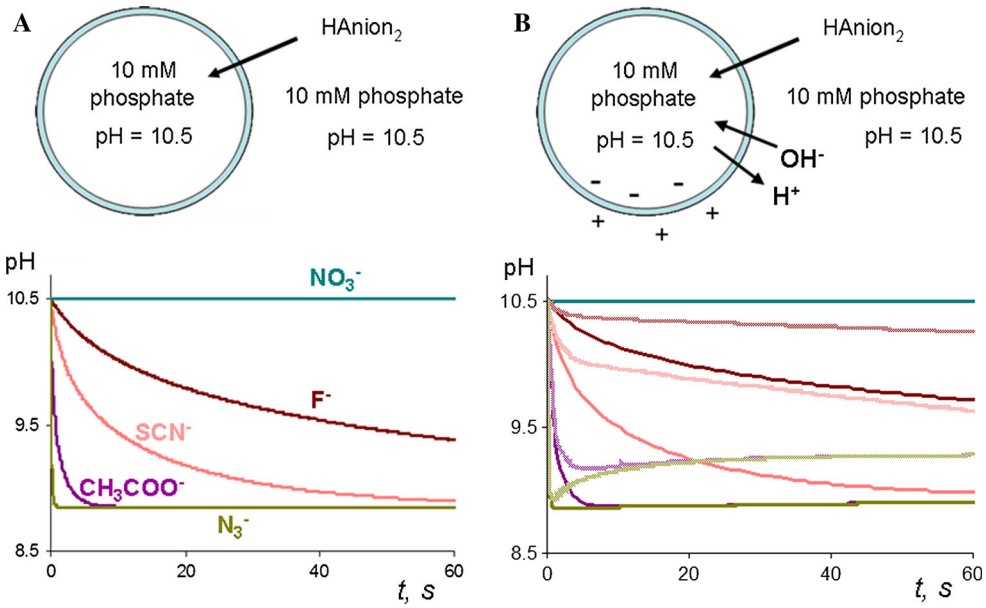

Fig. 3 Time-dependent changes in the intravesicular pH; $Anion_2^-$ is F^-, SCN^-, NO_3^-, or CH_3COO^-; $[Anion_2^-]_{OUT} = 100$ mM for SCN^-, NO_3^-, CH_3COO^-, and $[F^-]_{OUT} = 10$ mM; initial values of pH_{IN} and pH_{OUT} equal 10.5; 10 mM phosphate buffer. **a** $HAnion_2$ is the only permeant specie; **b** $P_{OH^-} = 10^{-9}$ m s^{-1}, $P_{H^+} = 10^{-3}$ m s^{-1} (mesh/light), and $P_{OH^-} = 4 \times 10^{-11}$ m s^{-1}, $P_{H^+} = 10^{-11}$ m s^{-1} (solid/dark) (Color figure online)

Supramolecular chemists like to operate with the initial rates, and therefore it is useful to consider the same phenomenon in the presence of the anionophore and to compare initial fluxes of ions H^+ and OH^- as well as anions of the salt.

First, we consider a classical anion receptor that utilizes only H-bonds or other weak contacts (anion–pi, halogen bonds) to coordinate ions in solution. Though the energy of hydration for Cl^- and OH^- is about the same and so as the permeability, it is important to keep in mind that almost all OH^- ions in solution are bound into the buffer and hence, the actual concentration even at pH 10 is only 0.1 mM. Consequently, the generated OH^- flux is tiny: 10^4 orders of magnitude (or more in a less basic medium) below the flux rates for anions of the salts, which concentrations are 100 mM in the intra- and extra-vesicular medium. In order to produce OH^- flux of the same magnitude as the two opposite fluxes of anions ("Cl^-/OH^- antiport" in the classical assay) this receptor would have to bind OH^- ions 10^4 times stronger (at pH 10) than it binds Cl^- so as P_{OH^-} increases accordingly (see also SI).

Contrastingly, if the anion transporter also acts as a protonophore the generated H^+ flux can be the same as the anion fluxes and even way above. The actual concentration of protons is also only 10^{-7} mM (at pH 7). However, the binding constant (K_b, M^{-1}) of a protonophoretic Bronsted base is in a range 10^4–10^9 in water ($pK_a = 4$–9) (Benz and McLaughlin 1983; Karlish et al. 1969; Schweigert et al. 2001), and that is many orders of magnitude higher than aforementioned non-covalent interactions with OH^- ion.

Finally, a chemist should expect that some lipophilic Lewis acids, like aforementioned metallophorphyrins, can selectively transport F^- over Cl^- and other relatively "soft" inorganic anions. These receptors, however, are also expected to selectively permeabilize biomembrane toward OH^-, which is also a very hard Lewis base. In order to answer the basic question about selectivity of these transporters (which could be related to chemical structure and binding affinities toward one or another inorganic anion) one should assess experimentally the anion permeabilities, absolute or the relative. The ability to distinguish between H^+ and OH^- flows experimentally will be advantageous for a researcher who has an outstanding opportunity to study classical Lewis acids as well as newly discovered pi–acidic receptors as a new and exciting form of the anion transporters, which both are thought to be strongly selective for strongly Lewis basic F^- (and OH^-) anions.

The Anion Selectivity: The Basic Anion Case

The modeling results illustrated in Fig. 4 have been obtained using Flowcharts C in Fig. 2. As in the classical assay, encapsulated anion is always the same, while extravesicular species are of all kinds. The transmembrane pH gradients are set ($\Delta pH = 1.38$) and so the process observed is "Cl^-/OH^- antiport".

As expected, regardless of rates of H^+/OH^- transport, apparent selectivity is always the same for non-basic anions: $Cl^- > Br^- > NO_3^- > I^- > ClO_4^-$. The intravesicular pH increases faster for stronger hydrated, hardly permeant anions and slower for weaker hydrated, relatively permeant species.

It is useful to reiterate the following. According to the classical technique, rates with which deprotonated form of pH-sensitive dye HPTS (excitation at 450 nm, emission at 510 nm) appears inside phospholipid spherules reflect the

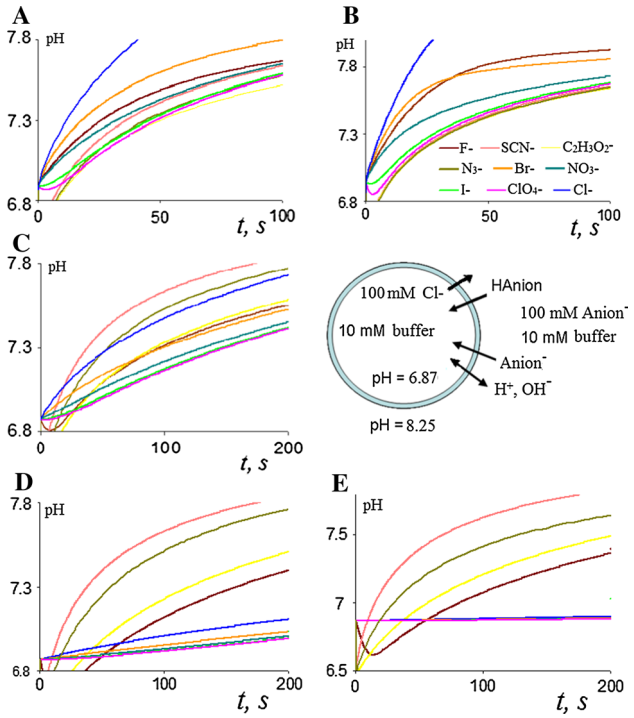

Fig. 4 Time-dependent change in the intravesicular pH in the classical assay; $[Cl^-]_{IN} = 100$ mM, $[Anion^-]_{OUT} = 100$ mM, $pH_{IN} = 6.87$ and $pH_{OUT} = 8.25$ are the initial values, 10 mM phosphate buffer; $Anion^-$ is as indicated, $P_{OH^-} = 4 \times 10^{-11}$ m s^{-1}. **a** $P_{H^+} = 10^{-3}$ m s^{-1}. **b** $P_{H^+} = 10^{-4}$ m s^{-1}. **c** $P_{H^+} = 10^{-5}$ m s^{-1}. **d** $P_{H^+} = 10^{-6}$ m s^{-1}. **e** $P_{H^+} = 10^{-7}$ m s^{-1} (Color figure online)

relative rates with which the extravesicular anions permeate across biomembrane. Thus, if the steady increase in emission at 510 nm is relatively fast than the extravesicular specie is relatively permeant. Using theory and modeling, we have recently shown that this relation is in fact, the opposite, namely the fastest increase in the intravesicular pH will be observed for the least permeant anion in this classical assay.

For instance, according to Matile and colleagues, the NDI-based anion pi-slides are Cl^--selective transporters with the anti-Hofmeister selectivity $Cl^- > Br^- > I^-$ (Gorteau et al. 2006). In our view, the selectivity is just Hofmeister-like—the anions permeate in the exact same order as they do across pure (unmodified with synthetic ionophores) lipid bilayer.

Another example is just recently described NO_3^--selective transporter. In a search for small moleculest would indeed overcome the Hofmeister bias Matile and colleagues discovered calix[4]pyrrole derivatives (Adriaenssens et al. 2013). In our view, however, the transport data should be read as follows: compound of interest does not selectively transport NO_3^-. Instead, it appears to have some selectivity for halides, Cl^- and Br^-, over the weaker hydrated NO_3^-. (Excluding basic F^- and CH_3COO^-, reported selectivity is $NO_3^- > Cl^- > Br^- > I^- > ClO_4^-$, in our view it is just reversed $NO_3^- < Cl^- < Br^- < I^- < ClO_4^-$).

It is relevant to note that it becomes quite challenging to access Cl^- versus NO_3^- selectivity using classical methods: lucigenin dye or Cl^--selective electrode. If we exclude the possibility of an artifact and assume reproducibility of the reported data, it would be safe to say that Matile and colleagues have successfully employed the advantages of the HPTS assay developed in the group to address the particular issue. Therefore, the findings someway coincide with the earlier report by Gale and colleagues for another calix[4]pyrrole, which suggested some $Cl^-(Cs^+)$ selectivity (Gale 2011; Tong et al. 2008).

In turn, in the case of basic anions, apparent selectivity is function of P_{H^+} as well as time moment when it is to be measured (Fig. 4). For instance, at $P_{H^+} = 10^{-3}$ m s^{-1} and $t = 100$ s it follows the order: $Cl^- > Br^- > F^- > NO_3^- \approx SCN^- > N_3^- \approx I^- \approx ClO_4^- > CH_3COO^-$. In turn, at $P_{H^+} = 10^{-5}$ m s^{-1} we have $SCN^- > N_3^- > Cl^- > CH_3COO^- > F^- > Br^- > NO_3^- > I^- \approx ClO_4^-$ at $t = 200$ s and $SCN^- > N_3^- \approx Cl^- > Br^- \gg CH_3COO^- \approx F^- > NO_3^- > I^- \approx ClO_4^-$ at $t = 50$ s. This inconstancy agrees well with the findings reported by Matile for the pi-slides and other synthetic molecules. According to the classical views, however, this particular feature (as well as modified and anti-Hofmeister selectivities) simply evidence strong interactions between the anion to be transported and the "exotic" receptors. For instance, "switch" in the selectivity pattern is expected to exist in the case of multi-ion hopping mechanism as in the pi-slides. In turn, the difference in activity and selectivity between different pi-slides (conjugates of several NDI units) and NDI monomers is simply due to the difference in the strength and specificity of the anion–pi interactions as well as transmembrane structures of the anion-conducting complexes (e.g., "the positively charge ammonium terminus increases the distance between the two rods inside the bilayer" (Dawson et al. 2010; Gorteau et al. 2006, 2007; Mareda and Matile 2009)).

Here, we argue that nature of these phenomena is quite simple, and the reported data can be explained by very basic properties of the anions and conjugate acids, plus different ability of various additives to facilitate H^+/OH^- translocation (anions of the weak acids, protonophoretic primary and secondary ammonium groups in the NDI rodes, etc.). It is also likely that rigid-rod molecules, like pi-slides, can disrupt the bilayer by changing its properties and so increase H^+/OH^- permeability without the necessity to bind these two ions inside biomembrane.

It is absolutely necessary to reiterate that despite of its simple origin, the dependence of pH_{IN} on time is complex. For example, if two compounds of interest have exactly the same anion transport properties but facilitate H^+/OH^- translocation

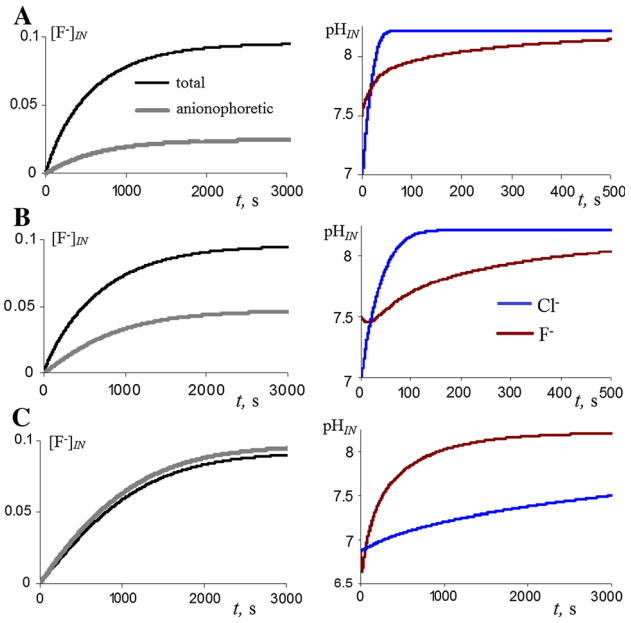

Fig. 5 Time-dependent change in the intravesicular pH in the classical assay; 10 mM phosphate buffer; $[Cl^-]_{IN} = 100$ mM, $[Anion^-]_{OUT} = 100$ mM, $pH_{IN} = 6.87$ and $pH_{OUT} = 8.25$ are the initial values; $Anion^-_{OUT}$ is either Cl^- or F^- as indicated. **a** $P_{H^+} = 10^{-3}$ m s^{-1}. **b** $P_{H^+} = 10^{-4}$ m s^{-1}. **c** $P_{H^+} = 10^{-6.5}$ m s^{-1}. (*Note* The amount of F^- that accumulates due to anionophoretic pathway exceeds the total amount of F^- in the vesicle (**c**) because fraction of F^- ion is released into the extravesicular solution by means of a weak acid mechanism) (Color figure online)

with different rates, one which is more efficient H^+/OH^- transporter will be more active Cl^-/OH^- antiporter as well. In contrast, if H^+/OH^- transport remains the same, one of the two which is to induce relatively slow anion exchange will appear as more active Cl^-/OH^- antiporter in these classical assays. When both, H^+/OH^- and anion transport are the unknowns, the outcome is unpredictable. Therefore, when it comes to comparative assessment of the compounds that form a group, pH_{IN} may not be particularly useful.

Finally, it is useful to note that the absolute rates discussed herein, at a glance, are about 2.5 orders of magnitude higher than those in the absence of additives, which induce HPTS response on the same time scale (Berezin 2009). In theory, selective transport of the particular inorganic anion should not necessarily result in a switch in the Hofmeister order. Whether there is a difference between values of the relative anion permeabilities, in the presence of a synthetic anionophore if compared to a pure lipid bilayer, has never been investigated.

Weak Acid Versus Anionophoretic Pathways: F^- Selectivity

The interplay between the weak acid pathway and the anionophoretic mechanism of F^- ion translocation is illustrated in Fig. 5. When H^+ is highly permeant, $P_{H^+} = 10^{-3}$ m s^{-1}, only about 25 % of the intravesicular fluoride accumulates due to transport by the anionophore (A). At $P_{H^+} = 10^{-4}$ m s^{-1} it rises to 50 % (B), while at $P_{H^+} = 10^{-6.5}$ m s^{-1} and below all the intravesicular F^- accumulates by means of the anionophoretic pathway (C). Interestingly, the apparent anion selectivity (measured at $t = 40$ s or above) switches from $Cl^- > F^-$ at $P_{H^+} = 10^{-4}$ m s^{-1} to $F^- > Cl^-$ at $P_{H^+} = 10^{-6.5}$ m s^{-1}. The apparent F^- selectivity of some NDI slides (halide VII selectivity sequence) (Gorteau et al. 2006), however, may well have a different origin, if measured in the very beginning of the transport process at $P_{H^+} = 10^{-4}$ m s^{-1} and above, when less than 2 % of the extravesicular F^- ion permeates across.

Most importantly, Fig. 5 illustrates that the anionophore-mediated transport of basic F^- can be studied using lipid vesicles. If the H^+/OH^- permeability of the reconstituted bilayer is low enough and the anion transporter does not act as a proton carrier (or permeabilize biomembrane toward H^+ by means of a different mechanism), the anionophoretic pathway dominates around and above pH neutral.

Strong Positive AMFE

When modeling the system which initially contains two different anions (Cl^- and I^-) in the extravesicular solution we were thrilled to observe a remarkably strong positive AMFE (Fig. 6). Underadditivity of "Cl^-/OH^- antiport" is a classical test to identify ion hopping in the synthetic systems. Strong positive AMFE in the $Cl^- + I^-$ (and $Cl^- + ClO_4^-$) mixtures was observed, for instance, in the case of NDI-based pi-slides and NDI monomers (Dawson et al. 2010; Gorteau et al. 2006, 2007; Mareda and Matile 2009). Consequently, a "multi-ion hopping" mechanism has been proposed. It is believed that two pi-acidic rods or four NDI monomers come together to form a transmembrane "suprastructure," the anion-selective passage way, where the anions strongly interact with pi–acidic surfaces and someway with each other and therefore, the positive AMFE is well expected.

A precise theoretical description of the physical picture behind these AMFE data seems to be of little relevance and so will not be discussed. It is important to highlight, however, that a small molecule that functions for instance, as an anion carrier with Hofmeister-like selectivity (and therefore, the anion translocation across the bilayer, facilitated by this receptor, can be perfectly understood as a passage over a single kinetic barrier) is well expected to exhibit strong-positive AMFE in the $I^- + Cl^-$ mixtures if tested in the classical supramolecular analytical assays developed by Matile and colleagues.

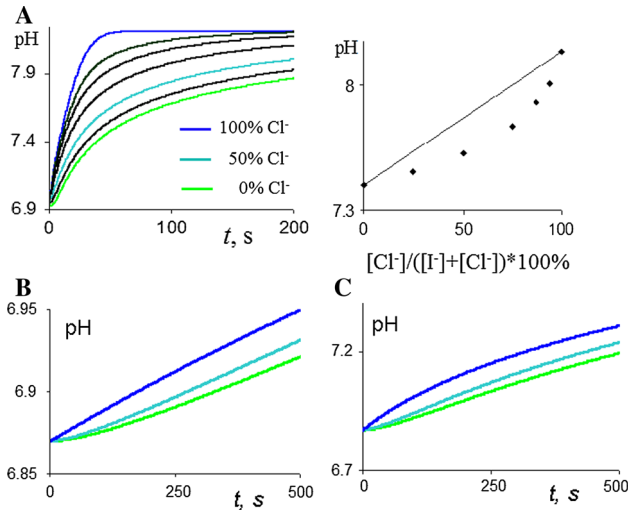

Fig. 6 Time-dependent change in the intravesicular pH in the classical assay and dependence of pH_{IN}, at $t = 45$ s on fraction of Cl^- in the extravesicular solution; $[Cl^-]_{IN} = 100$ mM, $[Cl^- + I^-]_{OUT} = 100$ mM, $pH_{IN} = 6.87$, and $pH_{OUT} = 8.25$ are the initial values; 10 mM phosphate buffer; $P_{OH^-} = 4 \times 10^{-11}$ m s^{-1}. **a** $P_{H^+} = 10^{-3}$ m s^{-1}. **c** $P_{H^+} = 10^{-7}$ m s^{-1}. **c** $P_{OH^-} = 10^{-6}$ m s^{-1} (Color figure online)

Finally, it is important to point out that Cl^-/OH^- antiport has been successfully applied to identify not only the anion selectivity sequences and positive AMFE but many other complex phenomena, which include negative AMFE (Hennig et al. 2009; Matile and Sakai 2007, 2012; Sakai and Matile 2006), Hill coefficient (self-assembly of the small molecules into channels and pores inside biomembrane) (e.g., Gorteau et al. 2006, 2007; Jentzsch et al. 2012; Mareda and Matile 2009; Muraoka et al. 2012), photosynthetic activity of the NDI rods (Perez-Velasco et al. 2008), sensitivity of macrodipolar transporters to membrane polarization (Hennig et al. 2009), function of the synthetic H^+ channel and pump (Bhosale et al. 2006; Weiss et al. 1997), and most recently, a new EC_{50}-based hopping mechanism of the anion translocation (Vargas Jentzsch and Matile 2013). Thus, in this latest study Matile and colleagues managed to identify so far probably the most efficient route to evidence ion hopping mechanism and weak interactions at work. A key to success is readily available libraries of the synthetic "monomers" and "oligomers." According to the authors, "presumable Cl^-/OH^- antiport," in combination with number of empirical relations, gives rise to unprecedented cooperativety coefficient ($m = 3.37$), proving a new, reliable, and readily available supramolecular analytical tool.

A theoretical analysis of these phenomena goes beyond the scope of the present study. Nevertheless, an interested researcher is encouraged to apply the chemical kinetics approach, theory and modeling, for independent explorations. For instance, the application of Hill analysis to the anion transport kinetics (both lucigenin- and HPTS-based approaches) requires a validation of the applicability from a theoretical viewpoint. The question whether a newly devised H-bond donor functions as a dimer or a monomer inside biomembrane is one of the most straightforward and essential in the field, particularly in light of development of the new CF therapeutics and tools for biomedical research.

Conclusion

In summary, we continue using theory only to study function of synthetic ion transporters, and the newly devised kinetic model adequately describes translocation of both, anions of the strong acids as well as basic anions, including strongly Lewis basic F^-.

In light of recent discoveries of the F^--selective channels in bacteria (Baker et al. 2012; Stockbridge et al. 2012) and a long-standing interest in F^--selective receptors and sensors (e.g., Badr and Meyerhoff 2005; Ke et al. 2012; Mascal et al. 2007; Wade et al. 2012; Woods et al. 2002), transport of this anion is of particular importance. Here, we show that in supramolecular field definition of F^--selectivity is delusive, nevertheless, because translocation of this basic anion can be easily studied using traditional liposome-based assays, the discovery of F^--selective synthetic carriers in a near future is well foreseen.

We also use theory and modeling to illustrate that both strongly positive AMFE and modified anti-Hofmeister selectivity may have a simple origin and complexity of these two phenomena is only apparent. We may reiterate that anion permeabilities used in the present study are just an arbitrary example, meaning that hydration energy determines the relative values. The interested researcher can use different P, m s^{-1} for anions of the salt or switch to the upper limit for the hydroxide ion, $P_{OH^-} = 10^{-9}$ m s^{-1} (here, we use the lowest reported value, $P_{OH^-} = 4 \times 10^{-11}$ m s^{-1}) to obtain the similar results and derive the same exact conclusions.

In turn, transport of OH^- versus H^+ ions is a complex question. Traditionally, synthetic anion transporters are thought to be OH^-/Cl^- antiporters and so as OH^--selective ["unidirectional ion pair movement (i.e., X^-/H^+) is osmotically disfavoured"] (Matile and Sakai 2007, 2012; Sakai and Matile 2006). On the other side, when it comes to transport of H^+, synthetic anionophores are thought to bind and transport across biomembrane an ion pair H^+Anion^- (Berezin 2013; Busschaert et al. 2011). The ambiguities that still surround synthetic anionophores at present led us to devise here a theory to distinguish between H^+ and OH^- translocation by the small synthetic molecules.

It is useful to reiterate that here we continue using GHK current equation to define the dependence of ion flux on the

electrical field. This implies solubility-electrodiffusion as a well-suited model (SI) (Hille 2001). Notably, for instance, halides are thought to permeate across lipid bilayer by means of solubility–diffusion mechanism, following the Hofmeister bias (Paula et al. 1998). In our case, it is an anion–receptor complex that partitions into biomembrane. The following step is electrodiffusion and the electrical potential changes linearly with distance. In turn, in the present study we have shown how GHK voltage equation (7) can be related to the ion transport in lipid spherules. It is remarkable that in the field, this particular equation has been used for decades to assess relevant phenomena in planar bilayer (selectivity of synthetic ion channels and pores) but never in liposomes.

At a glance, the partitioning-electrodiffusion model is not applicable if there is a significant occupancy of the binding site, meaning strong anion–receptor interactions and a second kinetic barrier for the anion exist (not only for the entry) (Hille 2001). Over the course of almost three decades, chemists created remarkable library of lipophilic membrane-active compounds with such binding sites in a row, which thought to function as entry or exit portals and (central) relays, using for instance, aforementioned crown ethers as a repeating unit (Fyles 2007; Gokel 2000; Gokel and Carasel 2007; Gokel and Daschbach 2008; Gokel and Mukhopadhyay 2001; Otis et al. 2013). It strikes that chemical kinetics has never been used to study those, though a theory used to study the Nature's ion channels has always been cited. Creation of the smart molecule with programmed properties, to mimic the natural phenomena, has always been a noble goal. By every measure, the chemical kinetics had to be taken along the way, but it was not. There are several factors to blame: (1) organic synthesis remains an easily available research tool; (2) the ion transport is a complex phenomenon and the researchers, with background and training in synthesis, were simply confused about theory and its applications; (3) in turn, even such complex phenomena as AMFE (interactions between ions of the same charge within biomembrane) could have been assessed using analytical tools developed by Matile and colleagues for lipid spherules; (4) and finally, there is only a handful of researchers that overlook advancements in the field. Not surprisingly, decades later, chemists are still not sure how to measure the anion selectivity in vesicles as an intrinsic property of the newly devised anion receptors.

The state of art is nicely combined in the conclusion: "On a structural level, the current examples (*of ion channels*) fall short, in some cases dramatically short, of even the simplest ion channels from Nature. But on a functional level, some of the activities and sophistication achieved with synthetic channels directly and quantitatively comparable to natural channels… (i.e.) As in all catalytic systems, the active structures and the molecular mechanisms of transport remain elusive. This is equally true of natural ion channels for which only a handful of structures are known, and the mechanistic insights they provide are ambiguous and incomplete. …. lack of mechanistic certainty need not inhibit the development of useful applications of artificial ion channels." (Fyles 2007). The Cl^--selective synthetic channel that regulates voltage-gated channels in vascular smooth-muscle cells (Li et al. 2009), synthetic hydraphiles of appropriate length that kill *E. coli* (Leevy et al. 2002), and scaffolded DNA origami that functions as a synthetic ion channel are just few such examples (Langecker et al. 2012). (The nano-assembly, composed of a single DNA strand of 7,249 bases length, staple strands and 26 nucleoside-cholesterol conjugates are the largest "molecule" (according to its molecular weight) reported so far to function as a synthetic ion channel.)

We have a different goal in mind: self-assembly and organic synthesis aim at the salient properties of the natural ion channels and transporters, such as activity, selectivity and in a long term, a remarkable combination thereof. We may reiterate that aforementioned lipophilic G-quadrulex (Arnaud 2005; Forman et al. 2000; Kaucher and Davis 2006; Kaucher et al. 2006) will be among the structures of particular interest, where the two can meet together. In our view, it is these particular salient properties that make the natural ion channels, exchangers, and co-transporters what they are, and so it has to be the same for the synthetic analog.

The ion hopping, selectivity, and other complex properties of the synthetic ion channels cannot be studied, using analytical techniques, as long as simplest anion carrier remains beyond the reach. A small synthetic anionophore meets the following two problems.

The ability to adopt a number of different conformations, depending on solvent polarity and solutes, is one of the key properties of the valinomycin. In complex with alkali metal, the amino acids' side chains point outwards, making it the most hydrophobic. In turn, metal-free receptor is less lipophilic which places this molecule away from the hydrophobic core, close to water-bilayer interface. Likewise, many synthetic cyclic (depsi)peptides seem to be structural as well as functional analogs of the natural product (Ovchinnikov et al. 1974). It should be expected that "valinomycin for anions" will share the same key properties and hence, may have a remarkably similar structure (cyclic peptide or peptidomimetic). In turn, its transport selectivity will simply be the result of its binding preferences, like in valinomycin. The first question to rise, however, is whether the observed selectivity can be directly related to the anion binding properties in the case of a classical synthetic anionophore. It appeals that a simple H-bond donor with rather rigid scaffold, which by

definition does not completely encapsulate inorganic ion, will also bind to a phosphate group in the lipid (contrastingly to valinomycin, which cannot encapsulate the choline heads). In our view, the competition between the two binding events—one to the surface and another one to the anion—in theory, might result in interesting twists in the anion selectivity pattern. Whether such phenomenon exists and plays a role in nature, in the living cell, is yet to be proven.

Moreover, when back in 2007 Davis and colleagues devised the new HPTS-based assay, it was discovered that in contrast to the TREN-based bis-catechol, the natural product prodigiosin displayed a remarkable selectivity for halides and nitrate over the much weaker hydrated perchlorate anion (Berezin and Davis 2007). And the very first question to rise was whether the nature of the observed phenomenon was in the intrinsic selectivity of this natural product or the lipid head groups played the key role. (The liposome-based assays and flowchart-based kinetic models appeal as an excellent combination of tools, which can be used to investigate this issue.)

Secondly, an interested researcher, willing to describe a novel anion receptor as a transporter in the biomembrane, will instantly get confused whether the molecule of interest functions as a channel or a carrier, forms a transient pore or employs other, more sophisticated mechanisms such as relays. Traditionally, the liposome-based techniques, easily available in organic laboratory, were thought to bring advantage over planar bilayer, providing deeper insights into selectivity, mechanism, and membrane-active structure of the synthetic transporters. More recently, however, a novel idea—that sophisticated electrophysiological techniques (to study electrogenic anion transport) can dissolve all the ambiguities—was generated. Accordingly, the mechanism just has to be probed the same exact fashion as the cation translocation by natural product valinomycin had been studied almost half a century ago. In turn, the study described herein should be classified as the one that strikes as utterly useless.

Here, we argue that liposomes, yet easily available in organic laboratory, is an essential tool. We disagree that it is simply a matter of choice—which method to choose. The planar and spherical lipid bilayers will complement each other, answering question that otherwise cannot be addressed.

This is not a secret that in a single living cell, like in any other living organism, a volume (size) as well as surface-area-to-volume ratio are crucial parameters for survival and function. This also makes the liposome an indispensable model of the living cell. To illustrate this point, let's assume that among two lead compounds of interest one turns out to be a "synthetic ion channel" (at the lowest concentration possible) while another one seems to be a carrier in the planar bilayer experiment (i.e., stepwise vs gradual increase in the ionic current). One should not be surprised if the first one yet yields a much better anion carrier (in terms of the relative activity and selectivity) when tested in liposomes (and so as in the living cell) than the second one. At a glance, such a statement contradicts the key postulate practiced in the field for three decades! In order to understand this complexity one should appreciate the physical phenomena behind the planar- and liposome-based studies in the case of small synthetic molecules, which in contrast to nature's transporters did not evolve naturally to function in highly selective and efficient manner. In other words, one may not apply empirical relations or theory-based equations without thinking of its nature and limitations, only pretending to be using analytical methods.

Due to the lack of opportunity to conduct any relevant experimental research (to pursue "valinomycin for anions," synthetic ion channels, the ion-pair transporters, F^- selective anionophores, etc.) we consider the two aforementioned problems a priority and will use chemical kinetics, theory and modeling, as a universal tool to investigate the issues.

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