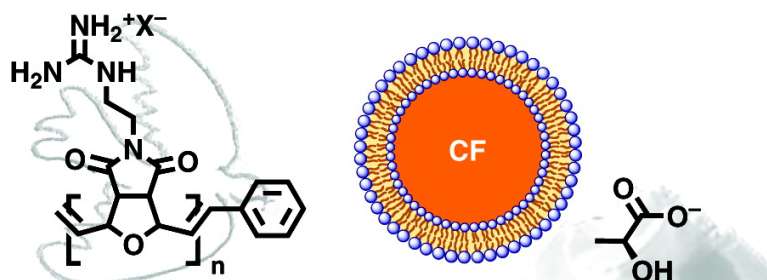Article

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Stimuli-Responsive Polyguanidino-Oxanorbornene Membrane Transporters as Multicomponent Sensors in Complex Matrices

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Abstract: We introduce guanidinium-containing synthetic polymers based on polyguanidino-oxanorbornenes (PGONs) as anion transporters in lipid bilayers that can be activated and inactivated by chemical stimulation. According to fluorogenic anion export experiments with vesicles, PGON transporters are most active in neutral bilayers near their phase transition, with EC_{50}'s in the nanomolar range. Six times higher effective transporter concentrations were measured with aminonaphthalene-1,3,6-trisulfonate than with 5(6)-carboxyfluorescein, demonstrating the importance of anion binding for transport and excluding nonspecific efflux. Negative surface potentials efficiently annihilate transport activity, while inside-negative membrane potentials slightly increase it. These trends demonstrate the functional importance of counterions to hinder the binding of hydrophilic counterions and to minimize the global positive charge of the transporter−counterion complexes. Strong, nonlinear increases in activity with polymer length reveal a significant polymer effect. Overall, the characteristics of PGONs do not match those of similar systems (for example, polyarginine) and hint toward an interesting mode of action, clearly different from nonspecific leakage caused by detergents. The activity of PGONs increases in the presence of amphiphilic anions such as pyrenebutyrate ($EC_{50} = 70$ μM), while several other amphiphilic anions tested were inactive. PGONs are efficiently inactivated by numerous hydrophilic anions including ATP ($IC_{50} = 150$ μM), ADP ($IC_{50} = 460$ μM), heparin ($IC_{50} = 1.0$ μM), phytate ($IC_{50} = 0.4$ μM), and CB hydrazide ($IC_{50} = 26$ μM). The compatibility of this broad responsiveness with multicomponent sensing in complex matrices is discussed and illustrated with lactate sensing in sour milk. The PGON lactate sensor operates together with lactate oxidase as a specific signal generator and CB hydrazide as an amplifier for covalent capture of the pyruvate product as CB hydrazone ($IC_{50} = 1.5$ μM).

Introduction

The activity of guanidinium-rich oligomers and polymers in biomembranes has attracted much scientific attention because of their biological importance and their complex behavior. In biology, these oligomers are usually arginine-rich peptides or proteins, such as heptaarginine **R7** or polyarginine **R72** (Figure 1). Prominent examples of arginine-rich proteins and their intriguing biomembrane activities include cell-penetrating peptides (CPPs),[1–4] such as HIV-TAT and many synthetic mimics,[1]

the voltage sensors of potassium channels,[2–5] and synthetic multifunctional pores.[6] The unique properties of arginine-rich oligo/polymers have made it difficult to understand their behavior on the molecular level, and as a result, these phenomena have sometimes been referred to informally as "arginine magic". Today, there is increasing evidence that the

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counterions of arginine-rich peptides and proteins account for much of this arginine magic.[2–5] The functional relevance of counterions originates in the weak acidity of the guanidinium group of the arginine side chain. Reduction of the pK_a to minimize charge repulsion by release of a proton, as in the more acidic ammonium-containing oligomers of lysine, is thus not possible for arginine-rich oligomers. As a result, tight binding of counterions remains as the only solution to compensate for the excess of positive charge.

Understanding the functional role of the resulting complexes is challenging because they are not only thermodynamically stable but also kinetically labile. This ability to rapidly exchange tightly bound counterions makes arginine-rich peptides and proteins exceptionally adaptable to many different environments. For example, to easily translocate across cell membranes, CPPs exchange their hydrophilic counterions with amphiphilic counterions to move across the bilayer membrane and then pick up their hydrophilic counterions again on the other side to exit the membrane.[2–4] The same concept of counterion-mediated function has been more recently suggested to account for the voltage gating of potassium channels with arginine-rich sensors.[5] Based on this model of the interplay between the lipid bilayer membrane, the guanidinium group, and the counterions, new functions of CPPs have been successfully produced in a rational manner. Examples include rapid cytosolic delivery of CPPs with counterion additives[2–4] and CPPs that can detect the activity of hyaluronidase inhibitors, which are potential targets for drug discovery.[7]

The ability to design simpler synthetic polymers that capture the biological activity of natural peptides remains an important scientific endeavor. Over the past few years, extensive effort has focused on synthetic mimics of antimicrobial peptides
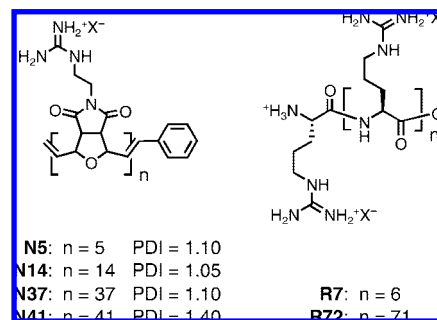

Figure 1. Structure of polyguanidino-oxanorbornenes (PGONs) **N** and cell-penetrating peptides (CPPs) **R** used in this study. All PGONs are mixtures of *cis*- and *trans*-alkenes and were prepared as TFA salts (X = CF₃COO⁻).

(SMAMPs).[8–10] These SMAMPs have focused almost exclusively on polycationic amine rather than guanidinium functionality. Expanding the range of peptide activity that can be successfully mimicked will enable new insight and potentially new opportunities for medical therapies, sensors, etc.[11]

Herein, we introduce synthetic guanidinium-rich polymers that, compared to polyarginine, are more hydrophobic and have a more shape-persistent scaffold, namely polyguanidino-oxanorbornene (PGON) transporters. We provide an extensive characterization of their membrane activity, including the dependence on pH, concentration, length, membrane fluidity, membrane potential, and surface potential. The global responsiveness of these transporters reveals significant differences when compared with similar systems. Their overall ability to respond to chemical stimulation by both activation and inactivation is similar to that of CPPs, although PGONs show different selectivities than CPPs. These findings imply that PGON−counterion complexes could act as multicomponent sensors in complex matrices,[12] a promising application of membrane transporters[13] that attracts current scientific attention but has so far been limited to synthetic multifunctional pores.[14,15] Lactate sensing in milk, with lactate oxidase for signal generation and Cascade Blue (CB) hydrazide for signal amplification,[15] is used to demonstrate their potential

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Scheme 1. Synthesis of PGON Polymers **N**[a]
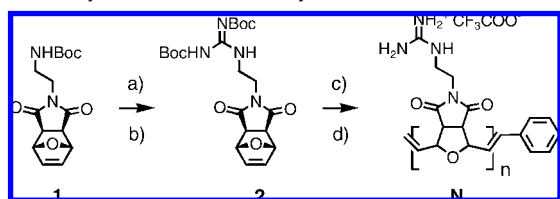
[a] Reagents and conditions: (a) TFA, CH_2Cl_2, 2 h, rt, quant. (b) N,N'-Di-Boc-1H-pyrazole-1-carboxamidine, TEA, (90% CH_3CN, 10% H_2O), 2 h, rt. (c) Grubbs catalyst, CH_2Cl_2, 0.5−2 h, rt. (d) TFA, CH_2Cl_2, 4 h, rt, quant.

in this area. We thus believe that PGONs could serve as an easily accessible membrane transporter, which complements the toolbox of available optical signal transducers in analyte sensing across membranes.[7,14,15]

Results and Discussion

Design and Synthesis. CPPs have been recently introduced as a readily available substitute for some sensing applications with synthetic multifunctional pores.[7] Similarly, PGON transporters can be synthesized in a less-demanding and straightforward manner (Scheme 1). The oxanorbornene compound **1**[9h] was guanidinylated in good yield to afford monomer **2**, which smoothly undergoes ring-opening metathesis polymerization (ROMP) using the third-generation Grubbs catalyst at room temperature in CH_2Cl_2. The degree of polymerization (DP) was controlled by varying the monomer-to-catalyst ratio, and the resulting Boc-protected polymers gave polydispersity indices (PDIs) between 1.05 and 1.40, based on GPC (polystyrene-calibrated) in THF. Once deprotected, these guanidinium-based PGON transporters were dissolved in water, filtered (0.45 μm pore), and then freeze-dried to afford soft pale solids which are readily soluble in buffer solutions.[16]

Activity. The activity of PGONs as well as their responsiveness to physical and chemical stimulation was determined in fluorogenic vesicle assays.[13–17] 8-Aminonaphthalene-1,3,6-trisulfonate (ANTS) and 5(6)-carboxyfluorescein (CF) were used as fluorescent probes in egg yolk phosphatidylcholine (EYPC) large unilamellar vesicles (LUVs), thus yielding
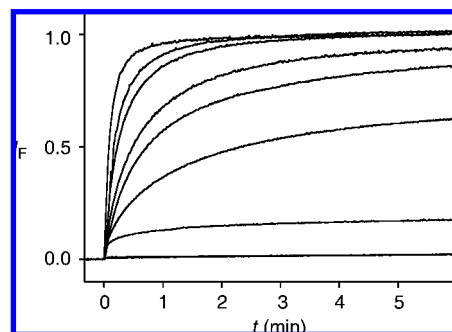

Figure 2. Dependence of transmembrane activity on the concentration of **N41**. Fractional CF emission intensity I_F ($\lambda_{ex} = 492$ nm, $\lambda_{em} = 517$ nm) is shown during the addition of **N41** (1 nM to 10 μM final concentration) to EYPC-LUVs⊃CF (50 mM CF, 10 mM Tris, 100 mM NaCl, pH 7.5, 25 °C, calibrated by final addition of Triton X-100).
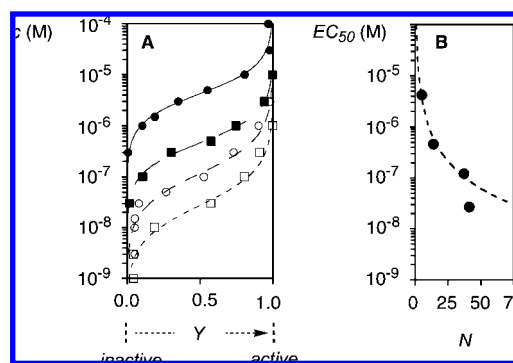

Figure 3. (A) Hill plot of PGONs **N5** (●), **N14** (■), **N37** (○), and **N41** (□) in CF vesicles with fit to eq 1. (B) EC_{50}'s of **N5**, **N14**, **N37**, and **N41** as a function of polymer length, i.e., the average number N of monomers per polymer, with fit to eq 2. Fractional activity Y compares fractional emissions I_F at given time after addition of the transporter (here 200 s after the start of the transport experiment; see Figure 2 for changes of I_F with time).

EYPC-LUVs⊃ANTS/DPX when loaded with ANTS and the quencher p-xylene-bis-pyridinium bromide (DPX), or EYPC-LUVs⊃CF when loaded with CF. In the ANTS/DPX assay, membrane activity is observed as an increase in ANTS emission due to the export of the cationic quencher DPX, the anionic fluorophore ANTS, or both from the vesicle and subsequent dilution into the surrounding media. In the similar CF assay, an increase in CF emission during CF export occurs because of the decrease in CF self-quenching upon dilution. The CF assay is more attractive for sensing, because of its higher sensitivity, whereas the ANTS/DPX assay is more attractive for characterization, because it is less dependent on pH and on the ion and size selectivity of the membrane transporters.[17]

The activity of PGON transporters increased with increasing concentration at constant vesicle concentration, as shown in Figure 2. From these curves, Hill plots were determined for the PGON transporters.[16–18] The obtained Hill plots revealed a nonlinear dependence of the fractional activity Y on the concentration c of monomeric macromolecules (Figure 3). Hill analysis using eq 1,

$$Y \propto (c/EC_{50})^n \tag{1}$$

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gave Hill coefficients n and effective concentrations EC_{50}, i.e., the concentrations needed to observe 50% activity. The consistently found Hill coefficients of $1 < n < 2$ revealed relatively poor cooperativity, although it cannot be excluded that the active transporter may consist of more than one monomer, and that the assembly of this active supramolecule is endergonic.[17,18]

Nevertheless, the EC_{50}'s decreased in a clearly nonlinear manner with polymer length (Figure 3B), and analyzing the data according to eq 2,

$$EC_{50} \propto N^{-p} \qquad (2)$$

revealed a quite remarkable polymer effect[19] ($p = 1.78$) that extended well beyond hydrophobic matching of polymer length and membrane thickness.[20] The EC_{50} of the longest **N41** was clearly better than expected from eq 2. The origin of this exception could be traced back to the high $PDI = 1.40$ of **N41** compared to the shorter polymers with $PDI \leq 1.10$ (Figure 1). The unusually high activity of **N41** thus originated presumably from the longer polymers in the less homogeneous mixture. For comparison, heptaarginine **R7** and polyarginine **R72** were inactive under these conditions without counterion activation (see below).[3]

Clearly higher EC_{50}'s were obtained with the ANTS/DPX assay in EYPC vesicles. For example, the $EC_{50} = 0.46 \pm 0.04$ μM of **N14** in CF vesicles increased 6 times to $EC_{50} = 2.8 \pm 0.3$ μM in ANTS/DPX vesicles. This dependence of transporter activity on the assay system was in support of specific anion transport and disfavored nonspecific anion leakage through major membrane defects. The lower activity with ANTS compared to CF was consistent with weaker binding of dianionic ANTS compared to trianionic CF to the polyguanidino transporter.

The pH profile of PGON transporters was obtained by adjusting the pH in the outside buffer. Increasing activity with increasing pH was observed with the CF and the ANTS assay. Both assays showed similar, slightly sigmoidal profiles, with the strongest increases occurring around pH 7.5 (Figure 4A). The observed independence on the assay system (CF or ANTS/DPX) ruled out that different protonation states of the fluorescent probes are responsible for the observed pH dependence. Far below the $pK_a \approx 12.5$ of guanidinium cations,[2–4] contributions from PGON deprotonation can also be readily excluded, even considering proximity effects. Most likely, fluorophore/OH$^-$ antiport is responsible for the observed pH dependence, with rate-limiting steps being OH$^-$ import below and fluorophore export above pH 7.5. This interpretation supports the postulated importance of anion binding. Similar interpretations could be formulated for phosphates as functional counterions (scavenged from the environment).[2–4,6a]

The dependence of the activity of PGON transporters on membrane fluidity was determined in dipalmitoyl phosphatidyl-
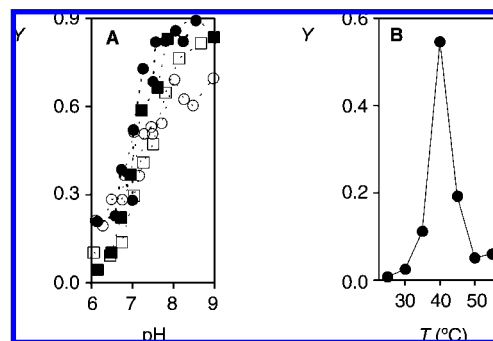

Figure 4. Dependence of PGON activity on (A) pH and (B) membrane fluidity. (A) pH profile of **N14** (○, □) and **N41** (●, ■) in EYPC vesicles loaded with CF (○, ●) or ANTS/DPX (□, ■). (B) Dependence of **N14** activity in DPPC vesicles on temperature (CF, pH 7.5).
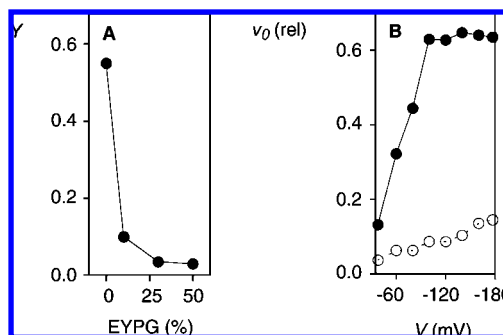

Figure 5. Dependence of PGON activity on (A) surface and (B) membrane potential. (A) Dependence of activity Y of **N14** on the mole fraction of EYPG in EYPC/EYPG-LUVs⊃ANTS/DPX. (B) Dependence of the initial velocity of change in emission of ANTS after addition of **N14** [1 μM (○) and 10 μM (●) final] to EYPC-LUVs⊃ANTS/DPX with valinomycin (0.6 μM), 100 mM internal KCl, and variable mixtures of iso-osmolar external KCl and NaCl.

choline (DPPC) vesicles. This is a common choice because the transition from gel to liquid-crystalline phase of DPPC vesicles occurs conveniently at 41 °C.[3,21] The highest activity of PGONs was found around the phase transition (Figure 4B). This behavior is different from that of CPP–counterion complexes[3] and many other synthetic and biological carriers, channels, and pores,[13] which are consistently most active in the fluid phase. Maximal activity located outside the fluid phase indicated that partitioning into the membrane does not limit activity.[17,22,23] Maximal activity located around the phase transition rather suggested that intermediate phases, such as the "ripple" phase, the membrane heterogeneity, or membrane defects that occur under these conditions, are important for activity.[21] The activity around the phase transition temperature in DPPC vesicles was comparable to that in the liquid phase in EYPC vesicles.

The dependence of the activity of PGON transporters on negative surface potentials was determined in mixed vesicles composed of neutral EYPC and anionic EYPG (phosphatidylglycerol).[2–4,23b] The presence of just 10% of EYPG in EYPC vesicles was sufficient to cleanly annihilate the activity of PGON transporters (Figure 5A). This behavior contrasts that of polyarginine, which is inactive in neutral EYPC vesicles without further additives but becomes highly

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active in the presence of increasing amounts of anionic EYPG.[2] Inactivation in anionic vesicles has, however, been observed for the lysine-rich pore melittin and for lysine-rich synthetic multifunctional pores.[2,23,24] While the latter showed less dramatic responses to the surface potential,[23] the response of melittin occurred in the same lipid ratio range (~10%) and was similarly intense.[2,23,24] The clear-cut inactivation of CF export by anionic lipids suggested that efficient multivalent binding of the cationic amphiphilic transporter to these anionic amphiphilic lipids may hinder counteranion exchange with hydrophilic anions. This interpretation was in agreement with results from the pH dependence and the dependence of Hill plots on the assay system (i.e., CF versus ANTS export). Inactivation by hydrophobic counterions confirmed that anion binding to and anion hopping along the polycationic transporter is essential for function. This behavior is clearly different from that of the more hydrophilic CPPs, which require hydrophobic counterion activators such as EYPG[2] to enable rather than inhibit the same anion-hopping mechanism across the lipid bilayer membrane.

To determine the dependence of the activity of PGONs on membrane potentials in EYPC vesicles, an inside-negative Nernst potential was applied.[22,23] To do so, the K^+ carrier valinomycin was added to vesicles with a transmembrane potassium gradient at concentrations high enough to produce a current but small enough to avoid immediate gradient collapse. Anion export and membrane potential were monitored simultaneously by double-channel experiments with internal ANTS (λ_{exc} = 353 nm, λ_{em} = 510 nm) and external safranin O (λ_{exc} = 522 nm, λ_{em} = 580 nm), respectively.

The dependence of anion transport with PGONs on the applied membrane potential was relatively weak (Figure 5B). The strongest changes were found for the initial rates of ANTS efflux at high concentrations of **N14**. This dependence was almost ohmic, clearly different from the non-ohmic behavior of biological (melittin, gating charge $z_g \approx 1.2$) and lysine-rich synthetic pores ($z_g \approx 0.85$).[23] Although only a weak dependence on the applied membrane potential was expected for a molecule lacking a permanent axial dipole moment,[23] inside-negative polarization should increase partitioning of cationic transporters into the lipid bilayer membrane. The observed relatively weak voltage sensitivity thus suggested that the positive charge of PGON transporters is counterbalanced by multiple counterion complexation (for example, with OH^-, as suggested from the pH dependence), resulting in transporter–counterion complexes with only weak positive total charge in solution. This finding supported the above-outlined model of thermodynamically stable but kinetically labile complexes and was in agreement with the results from surface potentials and Hill plots for CF versus ANTS.

Activators. The responsiveness of PGON transporters to activation was evaluated using the least active oligomer, **N5**. From the known[3] collection of amphiphilic counterion activators (Figure 6), only pyrenebutyrate **6** worked. Other amphiphilic counterions that were tested failed to activate **N5**. Examples include the homologous pyreneacetate **7** and pyrenehexanoate **8**, sodium dodecyl sulfate (SDS), sodium octyl sulfate (SOS), dodecyl phosphate, and cholate. The general role of amphiphilic counterion activators to increase binding to and accelerate the translocation across intact lipid bilayers is likely to apply to the activation of **N5** by pyrenebutyrate as well. With activity found for all pyrene homologues (**7**, EC$_{50}$ = 86 ± 3 μM; **6**,
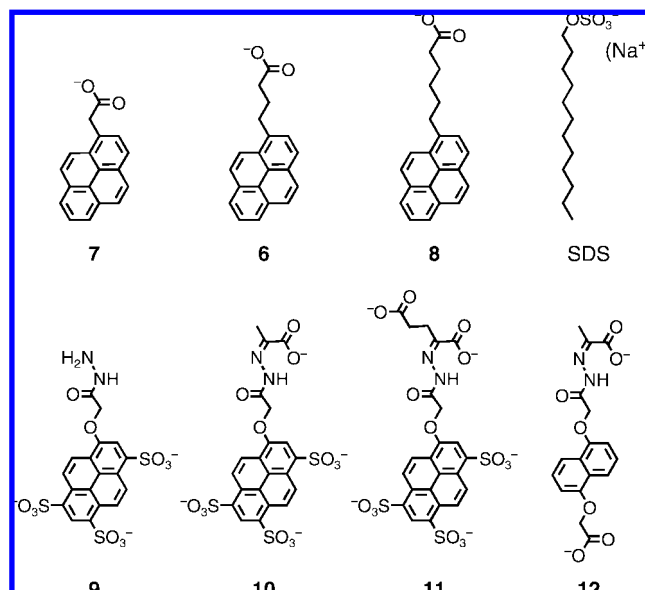

Figure 6. Structure of selected counteranion activators (top) and inactivators (bottom) for PGON and/or CPP transporters.

EC$_{50}$ = 44 ± 2 μM; **8**, EC$_{50}$ = 9.3 ± 0.7 μM), counterion activation of CPPs in EYPC vesicles was clearly less selective than with PGONs.[3]

The fluorescence spectra of pyrenebutyrate–PGON complexes in neutral water exhibited excimer emission similar to that of pyrenebutyrate–polyarginine complexes (and different from that of pyrenebutyrate–polylysine complexes).[2] For increasing PGON concentrations, the excimer emission appeared with an EC$_{50}$ = 2.3 ± 0.1 μM per polymer (94 μM per monomer) for **N41**. The EC$_{50}$ = 4.9 ± 0.3 μM (per polymer; 69 μM per monomer) for **N14** was very similar to and only slightly higher than that for polyarginine **R72** under these conditions (EC$_{50}$ = 0.7 ± 0.1 μM per polymer; 50 μM per monomer). The excimer emission decreased at higher polymer concentrations, presumably because of dilution of the bound pyrenebutyrates on the polymer backbone.[16]

Pyrenebutyrate was previously found to be the best to mediate CPP delivery to the cytosol.[4d] A combination of favorable activator–transporter and activator–membrane interactions, i.e., arene-templated ion pairing,[25] preferable binding at the membrane interface,[26] or matching lengths of lipid and activator, has been considered to account for the unique properties of pyrenebutyrate as activator.[2–4] However, the precise origin of certain exceptional properties of pyrenebutyrate as counterion activator, such as the selectivity of PGON activation, are unknown. Structural studies to investigate pyrenebutyrate–oligoguanidinium complexes in water and in bulk and lipid bilayer membranes on the molecular level are ongoing.

Pyrenebutyrate **6** activated PGON **N5** with an EC$_{50}$ = 70 ± 4 μM (Figure 7A, ○). The dependence on the sequence of addition was not very pronounced. Reversal from the routine sequence activator–vesicles–transporter to the sequence transporter–vesicles–activator gave an EC$_{50}$ = 40 ± 6 μM with clearly reduced cooperativity (Figure 7A, ●). This activator efficiency was in the range of the EC$_{50}$ = 44 ± 2 μM reported[4] for the activation of polyarginine **R72** with pyrenebutyrate **6**

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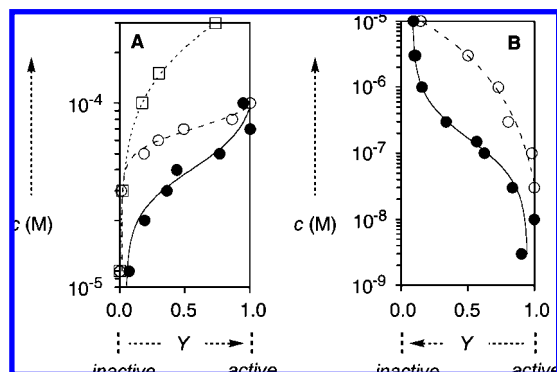
Figure 7. (A) Activation and (B) inactivation of PGON transporters. (A) Dependence of the anion transport activity of **N5** (○, ●) and **R7** (□) on the concentration of pyrenebutyrate **6**, with sequence of addition being activator−vesicles−transporter (○, □) or transporter−vesicles−activator (●). (B) Dependence of the activity of **N14** on the concentration of CB hydrazide **9** (○) and CB pyruvate **10** (●).

Table 1. Inactivators of PGON Transporters[a]

		IC_{50} $(\mu M)^{c}$	
entry	inactivator[b]	N14	N41
1	ATP	500 ± 100	150 ± 60
2	ADP	560 ± 140	460 ± 30
3	heparin	1.4 ± 0.1^{e}	1.3 ± 0.1^{e}
4	heparin (low MW)[d]	3.2 ± 0.3^{e}	1.0 ± 0.1^{e}
5	phytate	14 ± 3	0.4 ± 0.2
6	CB hydrazide **9**	26 ± 6	2.7 ± 0.7
7	CB pyruvate **10**	1.5 ± 0.1	0.4 ± 0.1
8	CB ketoglutarate **11**	1.0 ± 0.1	0.3 ± 0.04
9	DAN pyruvate **12**	>200	36 ± 4

[a] Determined from dose−response curves for fluorogenic CF export from EYPC-LUVs⊃CF as in Figure 7B. [b] See Figure 6; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate. [c] Inactivator concentration required for 50% inactivation of **N14** or **N41** (Figure 1), data ± SE. [d] MW, molecular weight; obtained by peroxidolysis from heparin, average MW = 3000 g/mol. [e] Based on an assumed molecular weight of 573.4 g/mol for the monomer, corresponding to a disaccharide composed of 2-*O*-sulfo-α-L-iduronic acid and α-D-*N*-sulfoglucosamine-6-*O*-sulfate.

and clearly better than the activation of heptaarginine **R7** with $EC_{50} = 197 \pm 8$ μM (Figure 7A, □).

Inactivators. The possibility to inactivate PGON transporters was investigated with the polymers **N14** and **N41**, which had intermediate and high activity, respectively (Figure 3). Hydrophilic counterion inactivators were selected depending on their usefulness in potential sensing applications.[14,15] ATP and ADP are important co-substrates that can be used in a very general manner to report on analytes that are compatible with signal generation by ATP-dependent enzymes. To take a simple example, the concentration of acetate in samples from the supermarket, such as rice vinegar, has been detected quantitatively by incubation with acetate kinase for selective signal generation and detection of the conversion of ATP into ADP with general optical signal transducers such as synthetic pores.[15] For this application, the signal transducer must be sufficiently sensitive to detect the presence of the ATP or ADP and must be able to discriminate ATP and ADP with a discrimination factor D as large as possible, but at least $D = 3$.[14,15] The inactivation of **N14** by both nucleotides occurred at very similar and rather high concentrations (Table 1, entries 1 and 2). With **N41**, the selectivity improved to $D = 3$ ($D = IC_{50}^{ADP}/IC_{50}^{ATP}$). This is sufficient for an enzyme assay but not for sensing in complex matrices,[7] and the relatively high $IC_{50} = 150$ μM clearly limits practical usefulness. The same is true for **R72** with

Scheme 2. Reaction Scheme for Lactate Sensing with Lactate Oxidase as Signal Generator and Hydrazide **9** as Reactive Signal Amplifier for Detection of the Otherwise Elusive Product Pyruvate as Hydrazone **10**
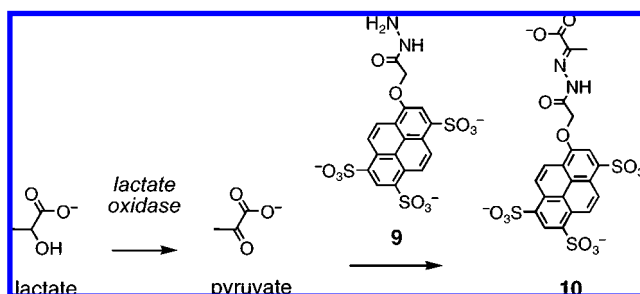
dodecylphosphate as counterion activator, which has $IC_{50}^{ATP} = 430$ μM and $D = 4$.[7] Only synthetic pores stand out so far in this regard, with performance as good as $IC_{50}^{ATP} = 1.2$ μM and $D = 15$.[14,15]

Heparin, a biologically important and chemically unique glycosaminoglycan that is widely used as an anticoagulant drug, inactivated both short and long PGONs **N14** and **N41** with excellent sensitivities in the low micromolar range, as expected on the basis of results with CPP−counterion complexes[2–4,7] and synthetic pores.[27] Comparison with a commercially available low-molecular-weight analogue, corresponding to a pentamer of disaccharide units, revealed that **N14** was capable of discriminating the low- and high-molecular-weight heparin with $D = 2$ (Table 1, entries 3 and 4). Taking into account that the three isoforms of heparinase commonly lead to further degradation down to disaccharide units, it is not unlikely that a simple heparinase assay could be developed, similar to the recently reported hyaluronidase assay with CPPs.[7]

Phytate (or inositol hexaphosphate, IP_6) is an analyte of importance with regard to the phosphate crisis and the sensing of the elusive IP_7.[14b] Phytate inactivated PGON transporters in a length-dependent manner (Table 1, entry 5). The best $IC_{50} = 400$ nM obtained with **N41** was clearly better than that obtained with **R72** ($IC_{50} = 5.4$ μM) but not as good as that obtained with synthetic pores ($IC_{50} = 45$ nM).[14b]

CB hydrazide **9** (Figure 6) is an important inactivator because it can serve as a reactive amplifier in sensing applications.[15] For example, to sense lactate in complex matrices, lactate oxidase is available as a specific signal generator, and the otherwise elusive product pyruvate can be covalently captured by hydrazide **9**, to give hydrazone **10** (Scheme 2). Lactate sensing thus requires the detection of hydrazone **10** without interference from hydrazide **9**. PGON transducers responded to **9** and **10** depending on the polymer length (Table 1, entries 6 and 7). Increasing sensitivity naturally coincided with decreasing selectivity. **N14**, with $D = 17$ at $IC_{50} = 1.5$ μM, exceeded synthetic pores in terms of selectivity ($D = 8.8$, $IC_{50} = 2.6$ μM) at comparable sensitivity,[15] while **N41** was even more sensitive at a comparable selectivity ($D = 6.8$, $IC_{50} = 0.4$ μM). Similarly promising results were obtained for CB ketoglutarate **11** ($D = 26$, $IC_{50} = 1.0$ μM for **N14**; $D = 9$, $IC_{50} = 0.29$ μM for **N41**), which is important for glutamate sensing.[15]

The π-basic dialkoxynaphthalene (DAN) amplifiers have been introduced for efficient inactivation of π-acidic synthetic pores by "adhesive π-clamping" with operational aromatic electron donor−acceptor (AEDA) interactions.[15] Applied to PGON transporters, DAN pyruvate **12** was essentially unable
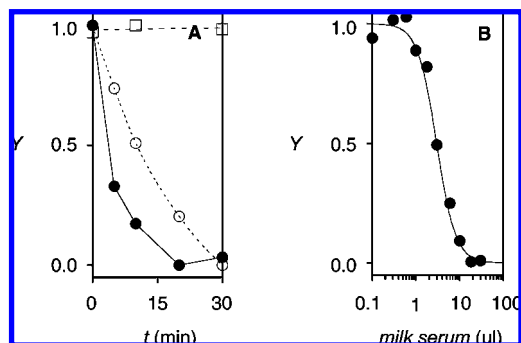
Figure 8. (A) Detection of enzyme activity and (B) sensing in complex matrices with PGON transporters. (A) Change in activity of **N14** as a function of the time of incubation of lactate with lactate oxidase (●, 2.5 units/mL; ○, 0.5 units/mL; □, 0 units/mL). Reaction mixtures were amplified with hydrazide **9** before detection of their ability to inactivate **N14**. (B) Change in activity of **N14** as a function the initial volume sour milk added after sample preparation (serum) and incubation with first lactate oxidase (2.5 units/mL) and then amplifier **9**.

to inactivate **N14** (Table 1, entry 8). Moreover, the $IC_{50} = 36\ \mu$M obtained for inactivation of **N41** was still 2.6 times higher than that obtained with synthetic pores ($IC_{50} = 14\ \mu$M).[15] As the formal monomer charge of π-acidic synthetic pores is 8+, these findings suggested that inactivation of synthetic pores by π-clamping with specific AEDA interactions is much more powerful than inactivation of PGON transporters by multi-ion pairing.

Detection of Enzyme Activity. To provide a meaningful example, the discrimination of CB hydrazide **9** and CB pyruvate **10** was selected to demonstrate the ability of PGON transporters to detect the activity of enzymes (Scheme 2).[14,15,27] The previously reported method was used with minor modifications.[15] In brief, lactate was first incubated with lactate oxidase and catalase to remove interference from hydrogen peroxide. To follow the conversion of lactate into pyruvate, aliquots were taken from the reaction mixture at meaningful intervals and incubated with CB hydrazide amplifier **9**. An aliquot of the obtained reaction mixture was then added to EYPC vesicles, and the appearance of fluorescence in response to the addition of **N14** was monitored. Decreasing fluorescence with increasing incubation time revealed the time course of the conversion of lactate into pyruvate, the latter being detected as inactivation of **N14** because hydrazone **10** inactivates better than hydrazide **9** (Scheme 2 and Figure 8A). The dependence of the reaction kinetics on the presence and the concentration of lactate oxidase demonstrated that **N14** indeed detects the activity of the enzyme.

Sensing in Complex Matrices. The ability of PGON transporters to act as optical signal transducers in sensing applications[14,15] was demonstrated with lactate oxidase as a signal generator and CB hydrazide **9** as signal amplifier. Following the procedures elaborated with synthetic pores,[15] milk serum was incubated first with lactate oxidase and then with amplifier **9**, and the effective volume of the obtained mixture to inactivate **N14** was determined (Figure 8B). Comparison of the obtained value with calibration curves multiplied with all dilution factors gave a lactate concentration of 72.2 mM in sour milk. This value was in the range determined previously with synthetic pores[15] and in excellent agreement with expectations from the literature.[28]

Conclusion

The activity of polyguanidino-oxanorbornenes (PGONs) as anion transporters in lipid bilayers has been characterized with

regard to the dependence on polymer length and concentration, pH, membrane fluidity, and surface and membrane potentials. Moreover, the responsiveness to amphiphilic counterion activators and hydrophilic counterion inactivators has been identified, characterized, and applied to the detection of enzyme activity and the sensing in complex matrices.

Comparison of the results with those obtained for similar systems, such as CPP−counterion complexes[2−4,7] and synthetic pores,[6,14,15,23,27] reveals clear differences, with some advantages and some disadvantages. The nonlinear increase of activity with polymer length provides a high membrane activity that can be obtained without counterion activators and much synthetic effort. However, the low micromolar EC_{50}'s achieved with PGONs are not in the range of activity of advanced synthetic pores. Different Hill plots for CF and ANTS transport, weak activation by membrane potentials, and strong inactivation by surface potentials all highlight the functional importance of specific anion exchange on and dynamic anion hopping along the polycationic polymer for transport and disfavor the existence of nonspecific leaks. Some characteristics, such as high activity around the phase transition and rapid inactivation by surface potentials, are clearly different from those of CPP−counterion carriers and point toward the occurrence of a distinct, interesting mechanism of action.

The general responsiveness of a guanidinium-rich anion transporter to activators and inactivators could be expected on the basis of extensive results with CPPs.[2−4,7] Nonetheless, the clear-cut discrimination between homologous activators such as **6**, **7**, and **8** is remarkable and so far not understood. The discrimination between CB hydrazide **9** and the corresponding hydrazones **10** and **11** is of immediate practical relevance for the detection of enzymatic activity and for sensing in complex matrices. The latter has been demonstrated with lactate oxidase to sense lactate in sour milk. Compared to the more hydrophilic CPPs such as oligo/polyarginines, the high intrinsic activity of PGONs allows reducing the complexity of the system, although also limiting the responsiveness to amphiphilic anions as activators other than pyrenebutyrate.

This first detailed report on the membrane activity of a synthetic mimic of polyarginine shows that the overall chemical structure and molecular backbone (more hydrophobic) can influence activity. With the wide variety of chemical structures available to the synthetic supramolecular chemist, it appears that synthetic polyguanidines will prove to be interesting new structures with strong relevance to membrane activity, sensing, molecular transport, and even potentially therapeutics.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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