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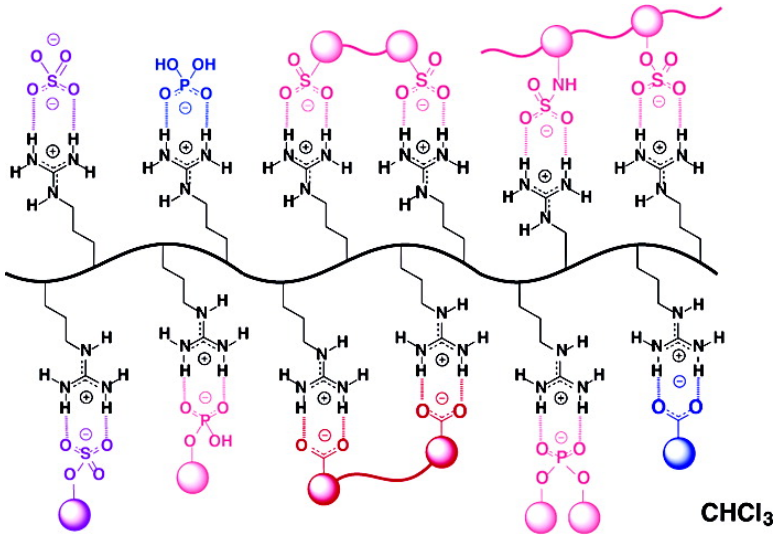

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Anion-Mediated Transfer of Polyarginine across Liquid and Bilayer Membranes

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Abstract: The accumulation of reports on the puzzling behavior of guanidinium-rich oligo/polymers in bilayer membranes, reaching from HIV-Tat-like (HIV Tat is the human immunodeficiency virus transactivator of transcription) translocation to selectivity and voltage-gating of ion channels, prompted us to investigate possible contributions from counteranions to these phenomena. We report that anion-mediated variability of charge and solubility makes guanidinium-rich oligo/polymers adaptable to many environments. For example, poly- and hexaarginine but not polylysine phase transferred from water into chloroform in the presence of amphiphilic anions such as monomeric sodium dodecyl sulfate (SDS), egg yolk phosphatidylglycerol (EYPG), cholesterol sulfate, pyrenebutyrate, and stearate. Hydrophilic anions with high affinity inhibited phase transfer of 5(6)-carboxyfluorescein (CF)−polyarginine complexes into bulk membranes (sulfate, adenosine 5′-triphosphate, adenosine 5′-monophosphate, heparin, and micellar SDS). At least binary anion cocktails were necessary to activate polyarginine as a carrier in bulk chloroform membranes. Refined combinations of EYPG, phosphate, and azide or TFA were found to maximize translocation of CF across bulk membranes by polyarginine. Polyarginine-mediated CF efflux from large unilamellar vesicles was best in the presence of EYPG in the bilayer as well as phosphate and TFA in the medium. Similar regulatory activities of several anions were in support of a common carrier mechanism for guanidinium-rich oligo/polymers in bulk and bilayer membranes. The identified activities of polyarginine in bulk and lipid membranes suggested that anion-mediated adaptability of the solubility of guanidinium-rich oligo/polymers cannot be ignored in studies on biological function. The infinite variability and dynamic nature of available regulatory anion cocktails may contribute to the elusive character of guanidinium-rich oligo/polymer function in biomembranes.

Introduction

In this paper, we describe simple phase-transfer experiments in liquid and bilayer membranes to experimentally support the previously stated[1] premise that at least some aspects of the unusual behavior of oligoarginines in biomembranes (i.e., "arginine magic") may originate from counteranion scavenging (Figures 1 and 2). One of the most notable examples of arginine magic is HIV-1 Tat, the human immunodeficiency virus type 1 transactivator of transcription. The potential of HIV-1 Tat transduction domain RKKRRQRRR to transport any conjugate across cell membranes is attracting considerable scientific attention.[2] The only structural requirement for cell-permeating activity seems to be the presence of multiple guanidinium cations. Other biological R-rich protein transduction domains (*Drosophila* Antennapedia transcription factor, herpes simplex virus type-1 VP22 transcription factor),[2] linear and branched oligoarginines,[3−5] D- and L,D-oligoarginines,[3−5] oligoarginine−stearate conjugates,[6] guanidinium-rich peptoids,[5] β- and α/β-peptides,[3,7,8] carbamate oligomers,[9] and peptide nucleic acids[10]

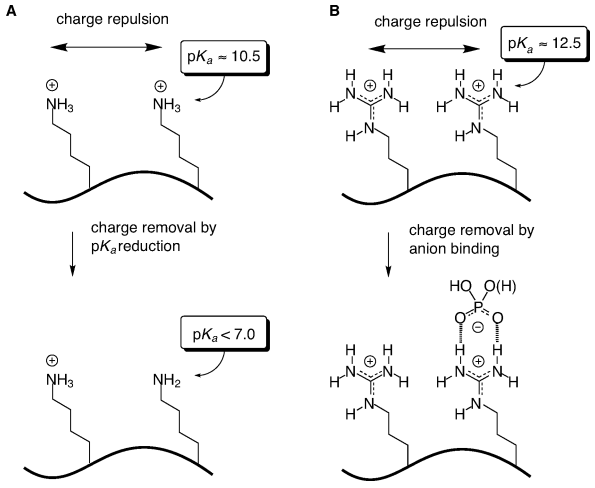

Figure 1. Proximity effects with (A) ammonium and (B) guanidinium oligomers enforce deprotonation and anion scavenging, respectively.

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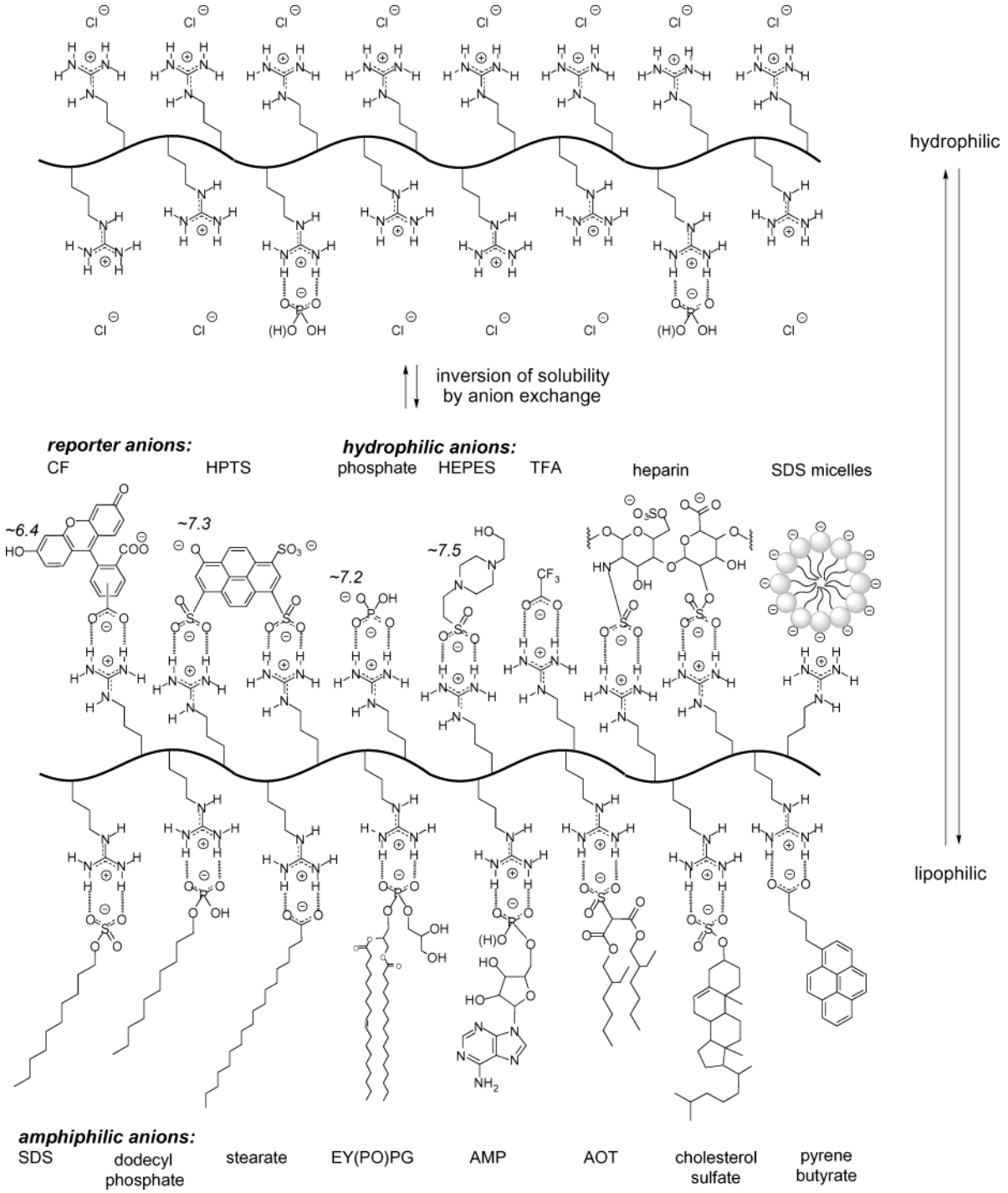

Figure 2. Concept of anion-mediated variability of the charge and solubility of oligo/polyguanidinium cations, illustrated schematically with molecular structures of some hydrophilic, amphiphilic, polymeric, and reporter anions explored in this study. The composition and charge of the depicted complexes are hypothetical; the indicated pK_a values refer to aqueous anions.

cross cell membranes rapidly. Fluorescein,[2] cyclosporin A,[11] caspase,[12] recombinase,[13] β-galactosidase,[14] liposomes,[15] DNA,[16] and nanomagnets[17] have been delivered as covalent conjugates with guanidinium-rich oligomers. Examples for noncovalent translocation with R-rich oligomers include DNA and heparan

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sulfate complexes.[18] Replacement of guanidinium by ammonium cations usually reduces translocation activity clearly; oligohistidines are less active as well.[8,19] Although the tolerance to structural modifications makes participation of highly specific protein receptors unlikely, it seems increasingly questionable that translocation of R-rich oligomers proceeds without involvement of endocytosis.[2,20−25] Compared to oligomers, polymeric and monomeric guanidinium transporters are less explored. Translocation of contrast agents (Gd chelates)[26] and DNA[27] complexed by polyarginine has been reported. Mono- or dimeric guanidiniums on cholate,[28,29] crown ether,[30] and cyclophane−rotaxane scaffolds[31] have been used to transport nucleotides,[28] amino acids,[29−31] and dipeptides[31] across bilayer and bulk liquid membranes.

The unusual behavior of oligo- and polyarginines in bilayer membranes is, however, not limited to translocation. Pores formed by amphiphilic guanidinium-rich polycations are cation rather than anion selective.[1,32−35] The partitioning of guanidinium-containing pores does not saturate, whereas that with ammonium-containing pores does.[36] Translocation of large arginine-rich paddles has been suggested very recently as the mechanism of voltage-gating of biological potassium channels.[37−39] This proposal has been criticized as energetically unrealistic because "lipid membranes are a forbidden zone for charged molecules".[40] Similarly unrealistic arginine magic may contribute to the modes of action of pore-forming R-rich natural antibiotics as well.[41−43]

Many examples for anion binding by mono-, oligo-, and polymeric guanidinium cations have been reported.[44−55] Guanidinium mono-, di-, and trimers have attracted much attention in molecular recognition because ion pairing can, *in principle*, be combined with preorganized hydrogen-bonding, cation−π, anion−π, and $\pi-\pi$ interactions.[44−47,28−31] Examples for anion binding by polymeric and particularly oligomeric guanidinium cations come from RNA,[48−51] DNA,[18,27,48,52] α-helix,[46] polysaccharide,[18,53] and dodecanoate micelle[54] recognition including studies on the mode of action of HIV-1 Tat. A leading example for anion binding by guanidinium-rich supramolecular polymers is phosphate scavenging by bilayer membranes composed of guanidinium amphiphiles.[55]

Anion binding by guanidinium-rich oligo/polymers differs from that by ammonium-rich oligo/polymers. The proximity of cations in these oligo/polymers results in charge repulsion (Figure 1). With nearby ammoniums, charge repulsion is minimized by reduction of pK_a values (Figure 1A).[56] A leading example for the importance of this proximity effect for function is the aldolases.[57] Because of the high pK_a of the guanidinium cation, the application of the same mechanism would be insufficient to enforce neutralization at pH ≈ 7. The only remaining solution to minimize charge repulsion in guanidinium-rich oligo/polymers in neutral water is anion scavenging (Figure 1B). The binding energy of guanidinium−phosphate pairs, for example, increases from $\Delta G = 0.9$ kJ/mol for monomers to a remarkable $\Delta G = 11-25$ kJ/mol for pairs in guanidinium vesicles.[55]

Anion scavenging by guanidinium-rich oligo/polymers will afford complexes with new physical properties (Figure 2). Full loading with monoanions or partial complexation with dianions will cause charge neutralization, extensive complexation with dianions charge inversion. Charge neutralization will increase the lipophilicity of guanidinium oligo/polymers. Amplification of this supramolecular lipophilicity by amphiphilic rather than hydrophilic counteranions is expected. Dynamic supramolecular adaptability of charge and solubility by anion exchange could, therefore, make guanidinium oligo/polymers either hydro- or lipophilic, depending on the environment. To test this hypothesis, we studied the ability of oligo- and polyarginines to transfer fluorescent "reporter anions" into hydrophobic solvents and across liquid and bilayer membranes in the presence of hydrophilic anions, amphiphilic anions, macromolecular polyanions, and supramolecular polyanions (Figure 2). We report

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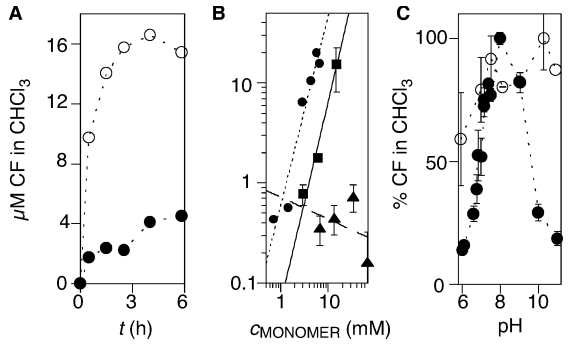

Figure 3. CF transferred from water (100 μM) into bulk chloroform membranes as a function of time (A, pH 7.4), the concentration of transporters (B, expressed as the concentration of monomeric amino acid residues, pH 7.4, 15 h), and pH (C, 20 h) in the presence of polyarginine (A−C, circles; 40 μM in panels A and C), hexaarginine (B, squares), and polylysine (B, triangles). Anions present were EYPG (A−C, filled symbols; 1 mM for panels A and C and 10 mM for panel B), SDS (A, C, empty symbols; 2 mM), sodium phosphate (A−C, filled symbols; 10 mM), HEPES (A, C, empty symbols; 10 mM), and NaCl (100 mM). The concentration of CF in graph C was normalized relative to the value obtained with EYPG and phosphate at pH 8.

that refined mixtures of anions can mediate phase transfer of oligo- and polyarginine into and across chloroform and across lipid bilayer membranes, and that these processes can be regulated—like "HIV Tat magic" [2,18,53]—with, e.g., glycosaminoglycans such as heparin.

Results and Discussion

Solubilizing Polyarginine in Chloroform. In a biphasic system of 0.2 mL of chloroform and 0.2 mL of water containing amphiphilic anions such as SDS (2 mM) and hydrophilic anions such as HEPES (10 mM, pH 7.4) and chloride (100 mM) but no polyarginine, "reporter anions" such as CF (100 μM) partitioned into the aqueous phase [SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; CF, 5(6)-carboxyfluorescein; see Figure 2]. Upon addition of polyarginine (40 μM), CF became detectable by the "naked eye" in the hydrophobic chloroform phase. Quantitative analysis by HPLC revealed that quite remarkable micromolar concentrations of CF were dissolved in chloroform under these conditions. The concentration of CF in $CHCl_3$ increased for hours after the addition of polyarginine (Figure 3A, empty circles). Similarly relevant, even slower phase transfer of CF into chloroform was observed when egg yolk phosphatidylglycerol (EYPG) and phosphate anions were used in place of SDS and HEPES (Figure 3A, filled circles). EYPG-mediated phase transfer of CF was more efficient with polyarginine than with hexaarginine; polylysine was inactive under all tested conditions (Figure 3B). Phase transfer of CF−SDS−HEPES−polyarginine complexes was nearly independent of pH (Figure 3C). The bell-shaped pH profile with CF−EYPG−phosphate−polyarginine complexes implied that contributions from the hydrophilic anion HPO_4^{2-} are not negligible (Figure 3C). This set of initial experiments provided clear-cut evidence that CF−polyarginine complexes can be solubilized in hydrophobic solvents such as chloroform in the presence of amphiphilic and hydrophilic anions.

a. Reporter Anions. In the presence of polyarginine and SDS, hydrophilic reporter anions CF and 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) could both be transferred into $CHCl_3$. Different from CF−polyarginine complexes, HPTS−polyarginine complexes did, however, not dissociate under RP-HPLC conditions. Unambiguous determination of the concentration of the reporter anion in $CHCl_3$ was, therefore, more problematic with HPTS than with CF. CF−SDS−polyarginine complexes in chloroform exhibited weak Cotton effects in the circular dichroism (CD) spectrum (Figure 4). With only one chiral compound involved, these CD Cottons provided direct experimental evidence that polyarginine is indeed dissolved in chloroform under these conditions.

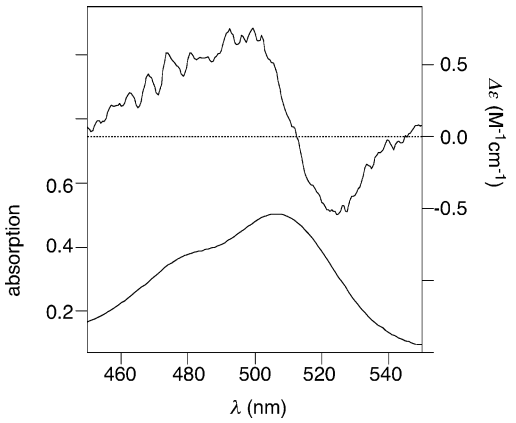

Figure 4. UV−vis (bottom) and CD (top) spectra of bulk chloroform membranes (2 mL) after phase transfer of CF from water (2 mL, 100 μM CF) in the presence of polyarginine (40 μM), SDS (2 mM), Tris (10 mM, pH 7.4), and NaCl (100 mM).

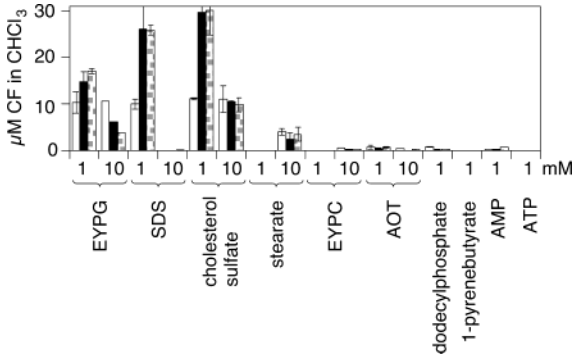

Figure 5. Screening of amphiphilic anions for the ability to mediate phase transfer of CF−polyarginine complexes from water (0.2 mL, 100 μM CF, 40 μM polyarginine) into bulk chloroform membranes (0.2 mL) in the presence of sodium phosphate (white), HEPES (black), and Tris buffer (dotted; all 10 mM, 100 mM NaCl, pH 7.4, 15 h, room temperature); see Figure 2 for the structures.

b. Amphiphilic Anions. Various amphiphilic anions were tested for the ability to mediate phase transfer of CF−polyarginine complexes into $CHCl_3$ (Figure 5). SDS, EYPG, and cholesterol sulfate showed significant activity; stearate was weakly active. Bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT), dodecyl phosphate, 1-pyrenebutyrate, adenosine 5′-triphosphate (ATP), adenosine 5′-monophosphate (AMP), micellar SDS (below), and zwitterionic egg yolk phosphatidylcholine (EYPC) did not mediate phase transfer of CF−polyarginine complexes. Possible explanations of inactivity include (reverse) micellization (AOT, >3 mM SDS), poor amphiphilicity (ATP, AMP), or displacement of CF to obstruct detection of polyarginine in chloroform (AOT, ATP, AMP, dodecyl phosphate, pyrenebutyrate).

Some of these uncertainties were clarified for pyrenebutyrate as an attractive example. Because of excimer emission in the

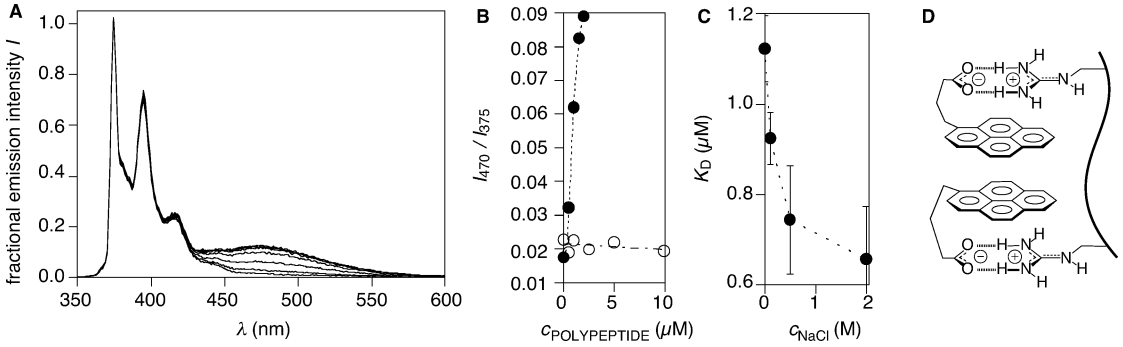

Figure 6. (A) Fluorescence emission spectra of pyrenebutyrate (120 μM) in water (10 mM sodium phosphate buffer, pH 7.4) in the presence of polyarginine (0−4 μM), excitation at 340 nm. (B) Relative excimer emission intensity (I_{470}/I_{375}) as a function of polyarginine (filled circles) or polylysine (open circles) concentration, conditions as in (A) plus 107 mM NaCl. (C) Dependence of K_D (± error estimated by curve fitting) on the concentration of NaCl (0, 0.107, 0.5, and 2 M). (D) Proposed structure of pyrenebutyrate−polyarginine complexes compatible with cation−π interactions, $\pi-\pi$ interactions, and excimer emission (schematic presentation with arbitrary distances).

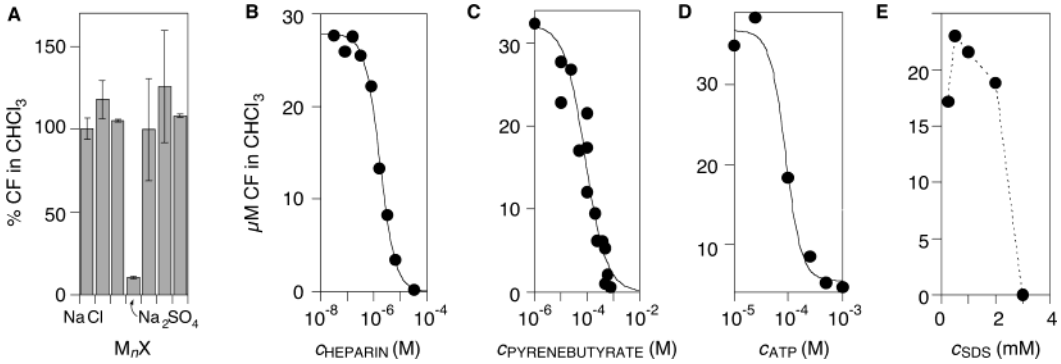

Figure 7. Screening of hydrophilic anions for the ability to mediate phase transfer of CF−polyarginine complexes from water. (A) Comparison of CF concentration in $CHCl_3$ with EYPG (1 mM) after phase transfer from water (100 μM CF, 40 μM polyarginine, 10 mM sodium phosphate, pH 7.4) containing, from left to right, 100 mM NaCl, LiCl, NaF, Na_2SO_4 (50 mM), NaOAc, NaTFA, and NaN_3. The data were normalized relative to those obtained with NaCl. (B) Concentration of CF extracted into $CHCl_3$ in the presence of increasing concentrations of heparin with a fit to the Hill equation (100 μM CF, 40 μM polyarginine, 2 mM SDS, 10 mM HEPES, 100 mM NaCl, pH 7.4). (C) Same for increasing concentrations of 1-pyrenebutyrate (conditions as in panel B with Tris instead of HEPES). (D) Same for increasing concentrations of ATP (conditions as in panel C). (E) Same for increasing concentrations of SDS (conditions as in panel B with varying concentration of SDS).

fluorescence spectra,[58] pyrenebutyrate−polyarginine complexes were readily detectable in water (Figure 6A). From the concentration dependence of excimer emission, dissociation constants (K_D) could be calculated. The global K_D values obtained by varying the concentration of polyarginine (Figure 6B, 0.92 ± 0.06 μM per polymer, 66.2 ± 4.3 μM per monomer) or pyrenebutyrate (not shown, 73.6 ± 2.5 μM) were in good agreement. Excimer emission could be observed in the presence of up to 2 M NaCl (Figure 6C). Slightly lowered K_D values at high ionic strength implied contributions from hydrophobic interactions (Figure 6D).[59] An analogous but steeper increase in affinity with ionic strength has been noted previously with a synthetic guanidinium receptor.[60] As in extraction experiments (Figure 3B), polylysine showed no activity with regard to pyrenebutyrate binding in water (Figure 6B, empty circles).

In biphasic systems, pyrenebutyrate partitioned into $CHCl_3$. However, pyrenebutyrate also inhibited solubilization of SDS−CF−polyarginine complexes in $CHCl_3$ (Figure 7C). This inhibitory activity could be interpreted only as phase transfer of pyrenebutyrate−polyarginine complexes into $CHCl_3$ in the presence or absence of other anions such as SDS and CF. The inactivity of stearate and acetate to inhibit CF extraction by SDS−polyarginine into $CHCl_3$ further supported the significance of cation−π and $\pi-\pi$ interactions to enforce binding of pyrenebutyrate to guanidinium cations in water and $CHCl_3$ (not shown). ATP, however, inhibited phase transfer of CF−polyarginine complexes such as pyrenebutyrate, but the produced ATP−polyarginine complexes remained too hydrophilic to transfer into $CHCl_3$ (Figure 7D).

c. Hydrophilic Anions. The influence of chloride [buffer tris(hydroxymethyl)aminomethane (Tris)], phosphate, and HEPES on the solubilization of polyarginine by amphiphilic anions in $CHCl_3$ was overall minor (Figure 5). The same was true for LiCl, NaF, NaOAc, NaTFA, and NaN_3 in place of NaCl (Figure 7A). Sulfate, however, inhibited the transfer of EYPG−CF−polyarginine complexes into chloroform. As with ATP (Figure 7D), high-affinity binding of sulfate to polyarginine may cause displacement of EYPG and CF to produce complexes that are overall too hydrophilic to partition into chloroform.

More potent inhibition of phase transfer of SDS−CF−polyarginine complexes into chloroform was observed with hydrophilic polyanions such as heparin (Figure 7B). An apparent global $K_D \approx 1.70 \pm 0.18$ μM ($K_D \approx 102$ μM per monomer) with Hill coefficient $n = 1.5 \pm 0.2$ was determined for heparin−polyarginine complexes.

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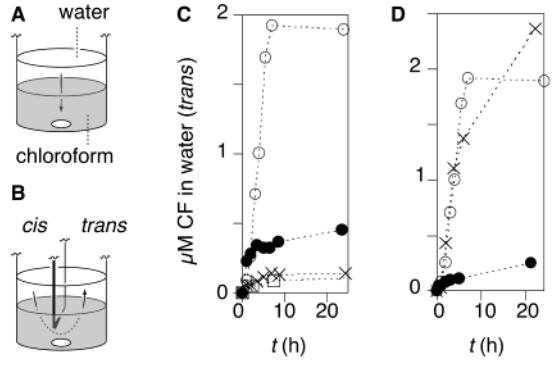

Figure 8. Screening of amphiphilic anions for the ability to mediate CF transfer across bulk chloroform membranes. (A) Experimental setup for phase-transfer experiments. (B) Experimental setup for U-tube experiments. Although this scaled-down version of a classical U-tube is not U-shaped, the term "U-tube experiment" is maintained for historical reasons, clarity, and convenience. (C) CF transferred from cis water (0.5 mL, 100 μM) across bulk chloroform membranes (3 mL) into trans water (0.5 mL) as a function of time in the presence of polyarginine (40 μM), EYPG (empty circles, 10 mM), SDS (filled circles, 0.33 mM), stearate (×, 10 mM), EYPC (squares, 10 mM), sodium phosphate (10 mM, pH 7.4), and NaCl (100 mM). (D) Same in the presence of polyarginine (40 μM), EYPG (10 mM), NaCl (100 mM), and 10 mM sodium phosphate (empty circles), HEPES (×), or Tris (filled circles, all pH 7.4) buffer.

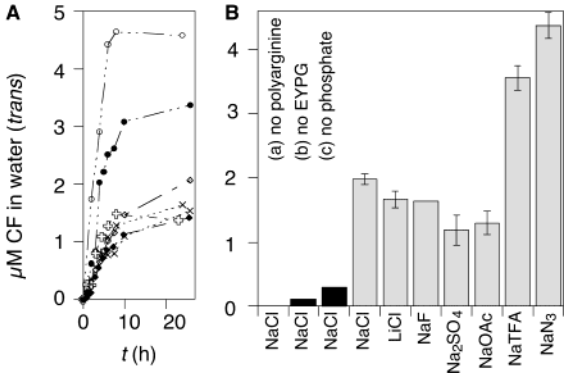

Figure 9. Influence of hydrophilic anions on CF transport by polyarginine across bulk chloroform membranes. (A) Concentration of CF transferred into trans water (see Figure 8B) as a function of time. (B) Concentration of CF in trans water after 25 h. Conditions: polyarginine (40 μM except for column a), EYPG (10 mM except for column b), sodium phosphate (10 mM, pH 7.4, except for column c), and 100 mM NaCl, LiCl, NaF, Na_2SO_4 (50 mM), NaOAc, NaTFA, and NaN_3.

The activity of SDS to phase transfer CF−polyarginine complexes into chloroform vanished abruptly around 3 mM (Figure 7E). This result can be explained by micellization of these amphiphilic anions into hydrophilic, supramolecular polyanions with amplified affinity for polyarginine.

Polyarginine as a Carrier in Bulk Membranes. Whereas phase transfer of polyarginine into bulk membranes requires binding of sufficiently hydrophobic anions at the interface to form neutral complexes as products, anion translocation across chloroform membranes involves both interfacial anion association and dissociation (Figure 8A,B). As described in the following, this additional step produced a remarkably different picture: Synergism between hydrophilic and amphiphilic anions became essential, a change that required identification and optimization of more complex anion cocktails.

In a typical "U-tube" experiment, polyarginine carriers as well as hydrophilic and reporter anions were added to the cis buffer, and concentration of the reporter anion in the trans buffer was monitored as a function of time (Figure 8B). Screening of amphiphilic anions in $CHCl_3$ at constant phosphate (10 mM hydrophilic anion), CF (100 μM reporter anion), and polyarginine (40 μM) concentration revealed high activity with EYPG. Some activity was observed with SDS (limited to low concentrations due to inactivation by micellization; see above); stearate and zwitterionic EYPC were inactive (Figure 8C). In sharp contrast to CF transfer into chloroform (Figures 5 and 8A), translocation of CF across chloroform required the presence of hydrophilic anions other than chloride (Figure 8B,D). This synergism was of sufficient interest to screen for refined mixtures beyond CF−EYPG−phosphate−polyarginine complexes (Figure 9). Azide and trifluoroacetate (but not acetate) were identified as the most powerful amplifiers of the translocation of CF across chloroform by EYPG−phosphate−polyarginine complexes. Illustrating the difference between the two processes, sulfate anions, identified as powerful inhibitors of CF transfer into chloroform, did not strongly affect translocation of CF across chloroform by EYPG−phosphate−polyarginine complexes (Figure 9B versus Figure 7A). These differences suggested that hydrophilic high-affinity anions may hinder CF transfer from cis water into chloroform (Figure 7A) but facilitate CF transfer from chloroform into trans water by ion exchange at the trans interface to either convert hydrophobic CF−polyarginine−anion complexes into hydrophilic ones or produce free CF (Figure 9B).

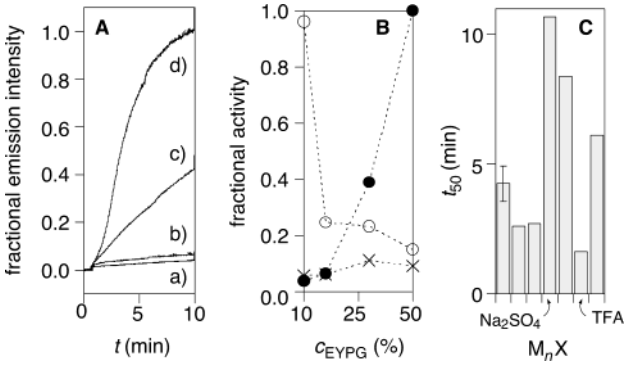

Figure 10. Screening of amphiphilic (A, B) and hydrophilic (C) anions for the ability to mediate CF efflux from LUVs ⊃ CF in the presence of polyarginine, melittin, and polylysine. (A) Fractional CF emission as a function of time during addition of polyarginine (250 nM after 0.5 min) to EYPC LUVs ⊃ CF containing (a) 0, (b) 10, (c) 30, and (d) 50 mol % EYPG. (B) Dependence of activity on EYPG concentration for polyarginine (250 nM, filled circles), melittin (200 nM, empty circles), and polylysine (10 μM, ×). (C) Time required for 50% CF release from EYPC/EYPG (1/1) LUVs ⊃ CF after addition of 250 nM polyarginine in the presence of 107 mM extravesicular NaCl, LiCl, NaF, Na_2SO_4 (53.5 mM), NaOAc, NaTFA, and NaN_3 (left to right). Constants: 13 μM lipid; extravesicular buffer, 10 mM sodium phosphate, 107 mM NaCl (A, B) or isoosmolar salts as specified (C), pH 7.4; intravesicular buffer, 50 mM CF, 10 mM sodium phosphate, 10 mM NaCl, pH 7.4.

Polyarginine in Anionic Vesicles. Polyarginine was already known to cause leakage from neutral[61] or anionic vesicles.[62] However, under the employed conditions, polyarginine was unable to mediate the release of the reporter anion CF from neutral large unilamellar vesicles (LUVs) composed of EYPC (Figure 10A, curve a). An increase of CF emission upon addition of polyarginine—implicative for efflux of self-quenched CF within LUVs[63]—became, however, easily observable as soon as amphiphilic EYPG anions were mixed into the neutral EYPC bilayer (parts A, curves b−d, and B, filled circles, of Figure

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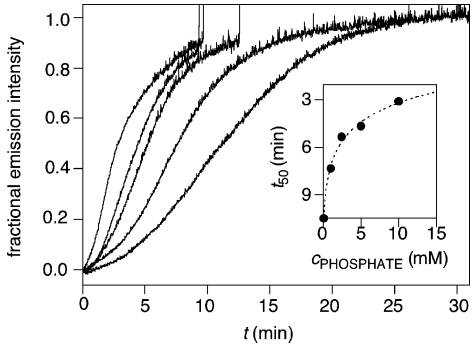

Figure 11. Fractional CF emission as a function of time after addition of polyarginine (250 nM) to EYPC/EYPG (1/1) LUVs ⊃ CF with an extravesicular ratio ($Na_mH_nPO_4$/HEPES) of, with decreasing activity, 1/0, 1/1, 1/3, 1/9, and ~1/99 at constant total concentration. Inset: Time required for 50% CF release as a function of extravesicular phosphate concentration. Conditions as in Figure 10 except the extravesicular buffer was $10 - x$ mM $Na_mH_nPO_4$, x mM HEPES, and 107 mM NaCl, pH 7.4.

10). Consistent with these results, increasing binding constants with increasing fraction of PG in small unilamellar vesicles (SUVs) were recently reported for HIV Tat.[64] Contrarily, decreasing activity with increasing fraction of PG in LUVs was observed with melittin,[65] a pore-forming toxin from bee venom (Figure 10B, empty circles). Polylysine was inactive under all conditions, also at concentrations exceeding that of active polyarginine up to 40 times (Figure 10B, ×).

Screening of hydrophilic anions revealed significant influences on the kinetics of CF transport (Figure 10C). Most results were consistent with those from extraction (Figure 7A) and U-tube experiments (Figure 9B). For example, extravesicular sulfate and TFA were identified as potent inhibitors and stimulators of polyarginine activity in EYPC/EYPG LUVs ⊃ CF (Figure 10C). These two anions already served well to regulate polyarginine activity in bulk membranes (Figures 7 and 9). Phosphate was as important for polyarginine activity in EYPC/EYPG LUVs ⊃ CF as it was in the U-tube (Figure 9B). Remarkably, exchange of extravesicular phosphate buffer by HEPES buffer more than tripled the halftime of polyarginine-mediated CF release (Figure 11). All kinetic traces exhibited a lag period after polyarginine addition followed by a smooth transition to maximal CF efflux (Figure 11). This sigmoidal time course was characteristic for an autocatalytic process. One interpretation of this finding is that accumulation of polyarginine on the membrane surface is necessary and rate limiting.

Comparative Summary and Discussion. Several amphiphilic anions are capable of mediating the solubilization of polyarginine and hexaarginine but not polylysine in chloroform (SDS, EYPG, cholesterol sulfate, pyrenebutyrate, and, less efficiently, stearate). Other amphiphilic anions do not mediate phase transfer of intact CF−polyarginine complexes (AOT, pyrenebutyrate). Hydrophilic anions with high affinity inhibit the same process (sulfate, ATP, AMP, heparin, and SDS micelles). These anions are inactive or inhibitory either because the resulting anion−CF−polyarginine complexes remain hydrophilic or—as confirmed for pyrenebutyrate—because anion−polyarginine complexes phase transfer into chloroform without reporter anion CF.

Table 1. Activity of Amphiphilic and Hydrophilic Anions To Mediate CF Transfer into and across Bulk Membranes as Well as CF Efflux from Vesicles in the Presence of Polyarginine[a]

	amphiphilic	hydrophilic I	hydrophilic II	into $CHCl_3$	across $CHCl_3$	from LUVs
01	EYPG	phosphate	TFA	+++++	+++++	++++++
02	EYPG	phosphate	azide	++++	++++++	+++++
03	EYPG	phosphate	Cl, F	++++	++++	+++++
04	EYPG	HEPES	Cl	++++	++++	+
05	EYPG	Cl	Cl	++++	+	nd
06	EYPG	phosphate	acetate	++++	+++	+++
07	EYPG	phosphate	sulfate	+	+++	++
08	EYPG	phosphate	heparin	−	nd	nd
09	SDS[b]	phosphate	chloride	++++++	++	nd
10	SDS[b]	HEPES	chloride	++++++	+	nd
11	stearate	phosphate	chloride	++	−	nd
12	EYPC[c]	phosphate	chloride	−	−	−

[a] Qualitative comparison of selected data from Figures 5 and 7−11. nd = not determined. [b] $c < 3$ mM (monomeric). [c] Zwitterion.

The requirement of at least one amphiphilic and—different from phase transfer *into* bulk membranes—also one hydrophilic high-affinity anion for phase transfer *across* bulk membranes resulted in high activity for refined cocktails such as CF−EYPG−phosphate−TFA−polyarginine complexes. The activity of phosphate to mediate translocation but not transfer is consistent with competitive binding of phosphate to guanidinium cations. HEPES is active in bulk membranes but not in LUVs (Table 1). However, the majority of activating and deactivating anions play quite similar roles in bulk and lipid bilayer membranes. This similarity may imply that polyarginine complexes can act as carriers in anionic bilayer membranes as they do in bulk membranes. Efforts to differentiate experimentally between carrier and other possible mechanisms of transport by polyarginine−anion complexes in spherical and planar bilayer membranes are ongoing.

It is impossible to explain HIV-Tat-like phenomena exclusively with anion-activated oligo/polyguanidinium carriers. It is, for example, difficult to imagine why oligo/polyguanidinium carriers should accumulate in endosomes or how an exclusive carrier mechanism could account for translocation of large macromolecules by short oligoarginines. In these situations, oligoarginines could function as phospholipid-specific anchors that partially penetrate the membrane, perhaps promoting changes in the membrane structure including formation of transient pores,[66] lipid domains, endocytosis, endovesiculation, and so on. Extrapolation to biological arginine magic remains, therefore, elusive beyond the notion that counteranions will always be there and matter. Sodium azide, for example, will not only serve as a biological probe but also produce new oligo/polyguanidinium complexes with new, activating or inactivating counteranions.

Conclusions

There is no problem to dissolve polyarginine in hydrophobic solvents as long as a synergistic mixture of hydrophilic and amphiphilic anions is present. There is also no problem to use the same, deceivingly hydrophilic polycation to mediate the translocation of anions across bulk and lipid bilayer membranes under the same conditions. Guanidinium-rich oligo/polymers, therefore, are both hydrophilic and lipophilic, depending on their counteranions.

The here-delivered experimental evidence for anion-mediated adaptability of the charge and solubility of guanidinium-rich

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oligo/polymers as well as its relevance for function demonstrates that counteranions cannot be ignored in the context of, e.g., HIV-Tat-like translocation or voltage-gating and selectivity of ion channels. The complex mixture of anions present in biological systems to possibly regulate guanidinium-rich oligo/polymer function makes more precise conclusions impossible.

Experimental Section

Materials and Methods. Poly-L-arginine (HCl salt, MW 14000, DP 72), poly-L-lysine (HCl salt, MW 22100, DP 134), SDS, AOT, Triton X-100, heparin sodium salt (17−19 kDa), ATP, AMP, buffers, and salts were purchased from Sigma. H-$(Arg)_6$-OH (TFA salt) was from Bachem. CF and stearic acid were from Fluka. HPTS was from Molecular Probes. EYPG and cholesterol sulfate were from Northern Lipids. EYPC was from Avanti Polar Lipids. 1-Pyrenebutyric acid was from Acros Organics. Mono-*n*-dodecyl phosphate was from Lancaster. Vesicles were prepared using a mini extruder with a polycarbonate membrane (Avanti Polar Lipids, pore size 100 nm). Fluorescence measurements were performed using a Fluoromax 2 (Jobin-Yvon Spex) equipped with a stirrer and a temperature controller (25 °C). HPLC was performed using an Agilent 1100 series apparatus with a photodiode array detector. CD spectra were obtained using a Jasco J-715 spectropolarimeter with a thermostated cell holder (25 °C). UV spectra were measured using a Varian Cary 100 Bio UV−vis spectrophotometer.

The "U-tubes" were house made. The setup was similar to that of Rebek and co-workers[67] with minor modifications (see Figure 8B). It consists of a small beaker (inner diameter 16 mm) with a wall in the middle, separating two aqueous phases named cis and trans for the sampling and receiving phases, respectively. The $CHCl_3$ layers below the cis and trans aqueous phases are connected by a small opening (height 10 mm) at the bottom of the wall separating the cis and trans buffers. The volume of the $CHCl_3$ layer was 3 mL; those of the cis and trans aqueous phases were 0.5 mL each.

Extraction Experiments. a. General Procedure. A solution of EYPG (1 mM) in $CHCl_3$ (0.2 mL) and an aqueous solution (0.192 mL, 10.4 mM $Na_mH_nPO_4$, 104 mM NaCl, 0.104 mM CF, pH 7.4) were placed in a vial and mixed vigorously. Then polyarginine (8 μL of a 1 mM aqueous solution) was added to the mixture. The final concentrations of each component in the aqueous solution were as follows: 40 μM polyarginine, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, 0.1 mM CF. Immediately after the addition of polyarginine, the biphasic solution was vortexed and placed for more than 15 h on a shaker (200 rpm) at room temperature. Then 20 μL of the $CHCl_3$ layer was injected into the HPLC instrument (column, Agilent Eclipse XDB-C8, 4.6 × 150 mm; mobile phase, linear solvent gradient of 1% aqueous TFA to TFA/CH_3CN = 1/99 over 10 min; flow rate, 1 mL/min; UV detection at 450 nm). The retention times of CF were 5.97 and 6.05 min (for the 5- and 6-isomers). Identical R_t values were found for uncomplexed CF in buffer. The concentration of CF in $CHCl_3$ was determined by comparing the integration value with the calibration curve. To ensure confidence, the experiments were performed at least twice, and average values ± error were plotted. Within the same set of parallel experiments, the data were consistent and the overall trends were highly reproducible. The day-to-day reproducibility of the final concentrations of extracted CF was poorer (see, e.g., Figure 7B−E). This could be due to differences in incubation time, temperature, anion scavenging, etc.

b. Extraction Kinetics (Figure 3A). Two vials were prepared. Vial 1 contained an aqueous solution (0.5 mL) of 40 μM polyarginine, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, and 0.1 mM CF, pH 7.4, on top of a solution of 1 mM EYPG in $CHCl_3$ (0.5 mL). Vial 2 contained an aqueous solution (0.5 mL) of 40 μM polyarginine, 10 mM HEPES, 100 mM NaCl, 0.1 mM CF, and 2 mM SDS, pH 7.4, on top of $CHCl_3$ (0.5 mL). The biphasic mixtures were prepared following the above general procedure; i.e., all components except polyarginine were placed in the vials and vortexed before and after addition of polyarginine. The two biphasic mixtures were stirred vigorously at room temperature, and 20 μL of the organic layer was taken every 1−1.5 h for HPLC analysis. The HPLC conditions were as described above.

c. Polypeptide Carriers (Figure 3B). Vials containing 0.2 mL of aqueous solution (10−80 μM polyarginine, 0.1−2.5 mM hexaarginine, or 50−500 μM polylysine, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, 0.1 mM CF, pH 7.4) on top of a solution of 10 mM EYPG in $CHCl_3$ (0.2 mL) were prepared following the general procedure. The concentration of CF in $CHCl_3$ was measured after 15 h of incubation. All data were from parallel experiments.

d. pH Dependence (Figure 3C). The following two sets of vials were prepared as described in Extraction Kinetics (Figure 3A): Vials of type 1 contained 0.2 mL of aqueous solution (40 μM polyarginine, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, 0.1 mM CF, pH 6−11) on top of a solution of 1 mM EYPG in $CHCl_3$ (0.2 mL). Vials of type 2 contained 0.2 mL of aqueous solution (40 μM polyarginine, 10 mM HEPES, 100 mM NaCl, 0.1 mM CF, 2 mM SDS, pH 6−11) on top of 0.2 mL of $CHCl_3$. The concentration of CF in $CHCl_3$ was determined by HPLC as in the general procedure after about 20 h of incubation.

e. Amphiphilic Anions (Figure 5). 1. EYPG, EYPC, AOT, Cholesterol Sulfate. Experiments were performed following the above general procedure using 1 or 10 mM EYPG, EYPC, AOT, or cholesterol sulfate in $CHCl_3$ (0.2 mL) below a buffer (0.2 mL; 40 μM polyarginine, 10 mM HEPES, Tris, or $Na_mH_nPO_4$, 100 mM NaCl, 0.1 mM CF, pH 7.4).

2. SDS, Pyrenebutyrate, AMP, ATP. SDS (1 or 10 mM), pyrenebutyrate, AMP, or ATP was included in the aqueous layer (0.2 mL; 10 mM $Na_mH_nPO_4$, HEPES, or Tris, pH 7.4, 100 mM NaCl, 0.1 mM CF, 40 μM polyarginine) on top of $CHCl_3$ (0.2 mL). Following the general procedure, the biphasic mixtures were vortexed before and after the addition of polyarginine, incubated, and analyzed by HPLC.

3. Stearate. A solution of stearic acid (1 or 10 mM) in $CHCl_3$ (0.2 mL) was prepared, and NaOH (0.2 or 2 μmol) was included in the aqueous layer (0.2 mL; 10 mM $Na_mH_nPO_4$, HEPES, or Tris, final pH 7.4, 100 mM NaCl, 0.1 mM CF, 40 μM polyarginine). Incubation and HPLC analyses were as in the above general procedure.

f. Hydrophilic Anions (Figure 7). 1. Figure 7A. Experiments were done following the general procedure using the indicated salts in place of NaCl. The aqueous layers (0.2 mL) on top of $CHCl_3$ (0.2 mL, 1 mM EYPG) consisted of 40 μM polyarginine, 0.1 mM CF, 10 mM $Na_mH_nPO_4$, pH 7.4, and 100 mM NaCl, NaF, LiCl, NaOAc, NaTFA, or NaN_3 or 50 mM Na_2SO_4.

2. Figure 7B. The aqueous layers (0.2 mL) on top of $CHCl_3$ (0.2 mL) consisted of 40 μM polyarginine, 0.1 mM CF, 2 mM SDS, 10 mM HEPES, pH 7.4, 100 mM NaCl, and 0.032−32 μM (0.1−100 units/mL) heparin.

3. Figure 7C. The aqueous layers (0.2 mL) on top of $CHCl_3$ (0.2 mL) consisted of 40 μM polyarginine, 0.1 mM CF, 2 mM SDS, 10 mM Tris, pH 7.4, 100 mM NaCl, and 1−1000 μM 1-pyrenebutyrate.

4. Figure 7D. The aqueous layers (0.2 mL) on top of $CHCl_3$ (0.2 mL) consisted of 40 μM polyarginine, 0.1 mM CF, 2 mM SDS, 10 mM Tris, pH 7.4, 100 mM NaCl, and 10−1000 μM ATP.

5. Figure 7E. The aqueous layers (0.2 mL) on top of $CHCl_3$ (0.2 mL) consisted of 40 μM polyarginine, 0.1 mM CF, 0.25−3 mM SDS, 10 mM HEPES, pH 7.4, and 100 mM NaCl. The data points in each figure were obtained from experiments performed in parallel. The data in different figures were not obtained in parallel experiments and cannot be compared quantitatively because of contributions from "day-to-day error".

CD Measurement of CF in $CHCl_3$ (Figure 4). "Large-scale" extraction of CF was done as described above using 2 mL each of an aqueous layer (40 μM polyarginine, 0.1 mM CF, 10 mM Tris, 100 mM NaCl, 2 mM SDS) and a $CHCl_3$ layer. The biphasic mixture was vigorously stirred for 15 h at room temperature and then centrifuged.

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The resulting clear organic layer was used for the CD measurement. The concentration of CF (9.7 μM) was determined by HPLC analysis as described above.

Pyrene Excimers (Figure 6). An aqueous solution of pyrenebutyrate was obtained by the addition of 1 equiv of NaOH to pyrenebutyric acid in water. A solution of pyrenebutyrate (120 μM) in a buffer (2 mL, 10 mM $Na_mH_nPO_4$, 0−2 M NaCl, pH 7.4) was placed in a cuvette. Then polyarginine (0−8 μL of a 1 mM aqueous solution) was added, and the solutions were gently mixed. Fluorescence emission spectra of the resulting solutions were measured with $\lambda_{ex} = 340$ nm. The obtained spectra were normalized relative to the maximal intensity at 375 nm. Normalized excimer emission intensities at 470 nm (I_{470}/I_{375}) for given NaCl concentrations were plotted as a function of polypeptide concentration and analyzed using eq 1 to obtain the K_D values ± error (Figure 6B). The calculated K_D values ± error were then plotted as a function of the concentration of NaCl (Figure 6C).

Data Analysis. Dissociation constants and Hill coefficients were calculated by fitting the data to eq 1, where I is the response (CF concentration or relative excimer emission intensity), I_0 the initial value, I_∞ the value at saturation, K_D the dissociation constant, c the concentration of the analyte, and n the Hill coefficient. Analyses were done with Kaleidagraph, version 3.5 (Synergy Software).

$$I = I_0 + (I_\infty - I_0)/[1 + (K_D/c)^n] \quad (1)$$

"U-Tube" Experiments. a. General Procedure. A solution of EYPG (10 mM) in $CHCl_3$ (3 mL) was placed in a U-tube (see the Materials and Methods and Figure 8B). The following cis and trans buffers were prepared and well mixed before addition on top of the organic layer: trans phase, aqueous buffer (0.5 mL; 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4); cis phase, aqueous buffer (0.5 mL; 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4) plus CF (0.1 mM) and polyarginine (40 μM). The organic layer was stirred at 700 rpm at room temperature. Aliquots (20 μL) were taken from the trans phase as a function of time and diluted to 2 mL with a buffer (10 mM $Na_mH_nPO_4$, 200 mM NaCl, pH 7.2). The concentration of CF was determined from the fluorescence emission intensity of the resulting solution at 517 nm (λ_{ex} at 497 nm) in comparison with the calibration curve (Figure 8C, empty circles). All U-tube experiments were performed at least twice.

b. Amphiphilic Anions (Figure 8C). 1. EYPC. Experiments were performed following the general procedure using EYPC instead of EYPG.

2. Stearate. To a solution of stearic acid (10 mM) in $CHCl_3$ (3 mL) was added NaOH (30 μL of a 1 M solution). The resulting mixture was vigorously mixed before use (general procedure).

3. SDS. The cis phase (0.48 mL; 0.104 mM CF, 10.4 mM $Na_mH_nPO_4$, 104 mM NaCl, pH 7.4) containing additional SDS (2.08 mM) was vortexed with $CHCl_3$ (0.5 mL). Then polyarginine (20 μL of a 1 mM aqueous solution) was added, and the mixture was vortexed again. Then both the organic and aqueous layers were added carefully to the U-tube containing $CHCl_3$ (2.5 mL) and the trans phase (0.5 mL) from the sampling side.

c. Hydrophilic Anions (Figures 8D and 9). 1. Figure 8D. Experiments were performed as in the general procedure with the following variation: In the cis and trans phases, 10 mM $Na_mH_nPO_4$ was replaced by 10 mM HEPES or Tris, all pH 7.4.

2. Figure 9A. Experiments were performed as in the general procedure with the following variation: In the cis and trans phases, 100 mM NaCl was replaced by 100 mM LiCl, NaF, NaOAc, NaTFA, or NaN_3 or 50 mM Na_2SO_4. Pertinent data from Figures 8 and 9A were summarized in Figure 9B. The data are shown as average values ± error in comparison to selected controls (a−c, general procedure without polyarginine, EYPG, or phosphate).

Vesicle Experiments. a. General Procedure. EYPC/EYPG LUVs ⊃ CF stock solutions [1.3 or 0.53 mM EYPC/EYPG = 1/1 (molar ratio); see below] were diluted with buffer (10 mM $Na_mH_nPO_4$, 107 mM NaCl, pH 7.4) to give ~13 μM lipid, placed in a thermostated fluorescence cuvette, and gently stirred. The time course of CF efflux was followed at $\lambda_{em} = 517$ nm ($\lambda_{ex} = 490$ nm) as a function of time during and after the addition of polyarginine (250 nM). Aqueous Triton X-100 (0.024%) was added at the end of each experiment. The data were normalized using eq 2, where I is the fractional emission intensity,

$$I = [(I_t - I_0)/(I_\infty - I_0)]/[(I_{max} - I_0)/(I_\infty - I_0)] \quad (2)$$

I_t is the fluorescence intensity at time t, I_0 is I_t before the addition of polyarginine (polylysine or melittin), I_∞ is I_t after lysis, and I_{max} is I_t at saturation for maximal activity before lysis (Figure 10A). From the normalized curves, the fractional activity Y (Y = maximal I before lysis) or t_{50} (the time required for 50% CF release) was recorded. The error level of two experiments performed within 8 h was as shown in Figure 10C, column 1.

b. Amphiphilic Anions (Figure 10A). The general procedure was repeated by adding 150 μL of 0.53 mM stock solutions of EYPC/EYPG LUVs ⊃ CF [EYPC/EYPG = 1/0, 9/1, or 7/3 (instead of EYPC/EYPG = 1/1)] to 1850 μL of buffer (10 mM $Na_mH_nPO_4$, 107 mM NaCl, pH 7.4, ~13 μM final lipid concentration).

c. Polypeptide Carriers (Figure 10B). The general procedure was repeated using polylysine (up to 10 μM) or melittin (200 nM) instead of polyarginine (250 nM).

d. Hydrophilic Anions (Figures 10C and 11). 1. Figure 10C. The general procedure was repeated using the following buffers instead of 10 mM $Na_mH_nPO_4$ (pH 7.4), 107 mM NaCl to dilute EYPC/EYPG LUVs ⊃ CF stock solutions (1.3 mM EYPC/EYPG = 1/1): 10 mM $Na_mH_nPO_4$ (pH 7.4), 107 mM LiCl; 10 mM $Na_mH_nPO_4$ (pH 7.4), 107 mM NaF; 10 mM $Na_mH_nPO_4$ (pH 7.4), 107 mM NaOAc; 10 mM $Na_mH_nPO_4$ (pH 7.4), 107 mM NaTFA; 10 mM $Na_mH_nPO_4$ (pH 7.4), 107 mM NaN_3; 10 mM $Na_mH_nPO_4$ (pH 7.4), 53.5 mM Na_2SO_4.

2. Figure 11. The general procedure was repeated using the following buffers instead of 10 mM $Na_mH_nPO_4$ (pH 7.4), 107 mM NaCl to dilute EYPC/EYPG LUVs ⊃ CF stock solutions (1.3 mM EYPC/EYPG = 1/1): 5 mM $Na_mH_nPO_4$, 5 mM HEPES, pH 7.4, 107 mM NaCl; 2.5 mM $Na_mH_nPO_4$, 7.5 mM HEPES, pH 7.4, 107 mM NaCl; 1 mM $Na_mH_nPO_4$, 9 mM HEPES, pH 7.4, 107 mM NaCl; 10 mM HEPES, (pH 7.4), 107 mM NaCl.

e. EYPC/EYPG LUVs ⊃ CF. LUVs composed of EYPC/EYPG with entrapped CF were prepared by the extrusion method. Solutions of EYPC and EYPG (10 mg, 1/0, 9/1, 7/3, or 1/1 molar ratio) in $CHCl_3$/MeOH (1/1) were dried under a stream of N_2 and then under vacuum (>2 h) to form thin films. The resulting films were hydrated using buffer A [10 mM $M_mH_nPO_4$, 10 mM MCl (M = Na or K), 50 mM CF, pH 7.4] for >30 min, freeze−thawed (5 times), and then extruded through a polycarbonate membrane (>15 times). Extravesicular buffer was exchanged using a Sephadex G-50 column equilibrated with buffer B (10 mM $Na_mH_nPO_4$, 107 mM NaCl, pH 7.4). Lipid concentrations were estimated from the amount of CF entrapped inside the vesicles. The found values were in agreement with earlier results from phosphate analysis (due to 10 mM phosphate in buffer; phosphate analysis did not give accurate results). Characteristics of the final stock solutions: 0.53 mM EYPC + EYPG; inside, 50 mM CF, 10 mM $M_mH_nPO_4$, 10 mM MCl (M = Na or K), pH 7.4; outside, 10 mM $Na_mH_nPO_4$, 10 mM NaCl, pH 7.4. More concentrated EYPC/EYPG LUVs ⊃ CF stock solutions (1.3 mM EYPC + EYPG, EYPC/EYPG = 1/1) were prepared following the above procedure starting with 25 mg of a 1/1 mixture of EYPC and EYPG. The identity of intravesicular cations M^+ (M^+ = Na^+ or K^+) had no effect on the transport kinetics.

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