Direct Observation of Anion-Mediated Translocation of Fluorescent Oligoarginine Carriers into and across Bulk Liquid and Anionic Bilayer Membranes

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The recent hypothesis that counteranion-mediated dynamic inversion of charge and solubility might contribute to diverse functions of oligoarginines in biomembranes was tested with two fluorescently labelled oligomers, FL-R_8, one of the most active cell-penetrating peptides, and its longer version, FL-R_{16}. We report evidence for counteranion-mediated phase transfer from water into bulk chloroform and anionic lipid-bilayer membranes as well as reverse-phase transfer from bulk chloroform and across intact lipid-bilayer membranes into water. The differences found between FL-R_8 and FL-R_{16} with regard to location in the bilayer and reverse-phase transfer from bulk and lipid-bilayer membranes into water implied that the reported results may be relevant for biological function.

Introduction

Guanidinium cations of oligoarginines bind very tightly to counteranions.[1,2] In clear contrast to the situation with ammonium cations in oligolysines (intrinsic $pK_a \approx 10.5$), the intrinsic $pK_a \approx 12.5$ of the guanidinium cation in oligoarginines is too high to allow partial deprotonation at pH 7 to minimize charge repulsion between proximal cations. Therefore, the thermodynamic stability of complexes between oligoarginines and their scavenged counteranions is expected to be high compared to that of monomeric guanidinium–anion complexes,[1–4] whereas their kinetic stability (i.e., the possibility of ion exchange) should not differ much. There is no doubt that the counterions of oligoarginines influence, if not determine their various, intriguing and significant functions in biomembranes. Because of rapid ion exchange, counteranion-mediated function is, however, a dynamic, adaptable phenomenon that is difficult to characterize and therefore often ignored.

Elaborating on the concept of counterion-mediated function, we recently found that there is no problem dissolving polyarginine–anion complexes in solvents as hydrophobic as chloroform as long as a synergistic mixture of hydrophilic and amphiphilic anions is present.[1] We further found that the same, deceivingly hydrophilic polycation can mediate the translocation of anions across bulk and lipid-bilayer membranes under comparable conditions.[1] These results suggested that counteranions of guanidinium-rich oligo-/polymers cannot be ignored in the context of, for example, the translocation of cell-penetrating peptides (CPPs)[1,5–29] or voltage gating[30–32] and selectivity[2] of ion channels and pores. However, extrapolation of these findings with polyarginines to the function of biologically relevant oligoarginines was not straightforward as, in the former case, polyarginine was indirectly detected by bound reporter anions such as carboxyfluorescein. To gain insight into the importance of counterions for the function of oligoarginines, herein we explore anion-mediated phase transfer of covalently fluorescein-labelled oligoarginines of biological relevance, that is, octaarginine as one of the most efficient and typical oligoarginine CPPs (FL-R_8), and its longer version, the arginine 16-mer (FL-R_{16}) (Figure 1).

It has recently been suggested that endocytosis contributes to the cellular uptake of oligoarginine CPPs such as fluorescein-labelled FL-R_8 and FL-R_{16}.[7,23] However, a nonendocytotic pathway has not been completely excluded as an internalization pathway as shown, for example, in the internalization of CPPs at 4 °C[17,24,25,27] as well as substantial voltage-dependence in model vesicles[9] and, remarkably, also in cells.[14b] In addition, even when delivered into cells by endocytosis, CPPs still have to cross the endosomal membranes to serve as bioactive molecules in the cells, and this mechanism is still unclear. Considerable differences in the manner of cellular uptake and cytosolic release between the arginine 9-mer and 15-mer have also been pointed out.[19]

Focusing on the concept of anion-mediated function, we here report that oligoarginines can be transferred almost completely from water into chloroform in the presence of amphiphilic anions like egg-yolk phosphatidylglycerol (PG), bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT), cholesterol sulfate (CS) or monomeric sodium dodecylsulfate (SDS; Figure 1C).[27]

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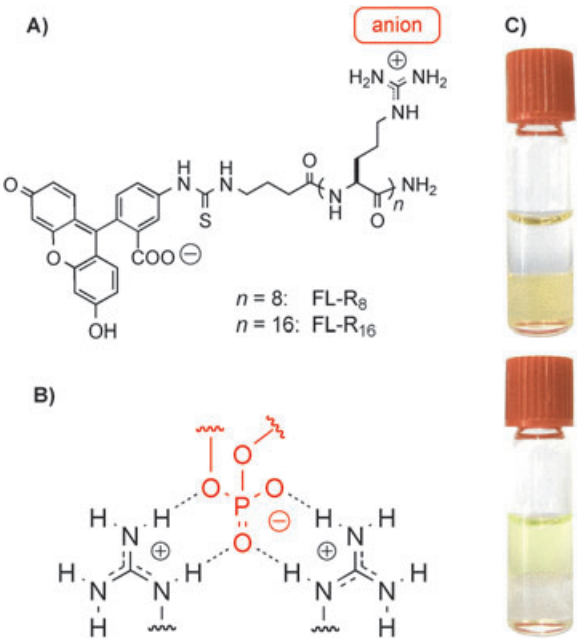

Figure 1. *Anion-mediated transfer of (FL-R_n)–anion complexes. Structures of A) FL-R_8, FL-R_{16} and B) 2:1 guanidinium/phosphate complex. C) Typical results of extraction experiments with FL-R_8 (25 μM), PG (top, 0.25 mM; bottom, 0 mM), $CHCl_3$ (0.4 mL), and a buffer (0.4 mL, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4).*

This phase transfer can be inhibited by hydrophilic polyanions like heparin[21,22] and micellar SDS. Counteranion-mediated (sulfate, heparin) reverse-phase transfer of PG complexes from chloroform into water—mimicking the intracellular release from biomembranes—is possible with (FL-R_8)–anion complexes but not with (FL-R_{16})–anion complexes. According to shifts in FL emission, both oligomers preferentially locate near the interface of anionic PG/PC (egg-yolk phosphatidylcholine) vesicles. Biphasic kinetics for removal from PG/PC vesicles with heparin provide direct evidence for the translocation of intravesicular (FL-R_8)–anion complexes but not (FL-R_{16})–anion complexes across intact spherical bilayers. The conditions for this direct observation of anion-mediated translocation differ from the recently reported movement of cationic oligomers into polarized vesicles (inside negative) doped with divers anionic lipids.[9] Support for transmembrane motion under comparable conditions is available from isothermal calorimetry for Trojan peptide penetratin,[13] although this situation is somewhat different.

Results and Discussion

The objective of this study was to obtain insights into the relevance of anion-mediated change in solubility and charge of oligoarginine carriers to function. To do so, four distinct phase-transfer processes were investigated separately with fluorescently labelled model oligomers with one of the typical CPPs (FL-R_8) and its longer version (FL-R_{16}), namely:

1) phase transfer from water into bulk chloroform membranes,
2) reverse-phase transfer from bulk chloroform membranes into water,
3) phase transfer from water into lipid-bilayer membranes, and
4) reverse-phase transfer from and across lipid-bilayer membranes into water.

Anion-mediated phase transfer of FL-oligoarginines from water into bulk chloroform membranes

In a biphasic system of 0.4 mL chloroform and 0.4 mL water containing amphiphilic anions like PG (250 μM), hydrophilic anions like phosphate (10 mM, pH 7.4) and chloride (100 mM), anion complexes of fluorescently labelled octaarginine FL-R_8 (25 μM) transferred completely into the organic phase (Figure 1C). Unambiguous quantification of the FL concentration in the organic layer by reverse-phase (RP) HPLC failed because only partial release of the amphiphilic PG counteranions from (FL-R_n)–anion complexes under these conditions resulted in extensive peak broadening (Figure 2C). The less satisfactory analysis from calibration curves of the FL emission was, however, unproblematic with aqueous (FL-R_n)–anion complexes. Although the dependence of the emission intensities on the concentration of hydrophobic (FL-R_n)–anion complexes diluted in MeOH was linear, we decided to report relative intensities only to avoid overinterpretations.

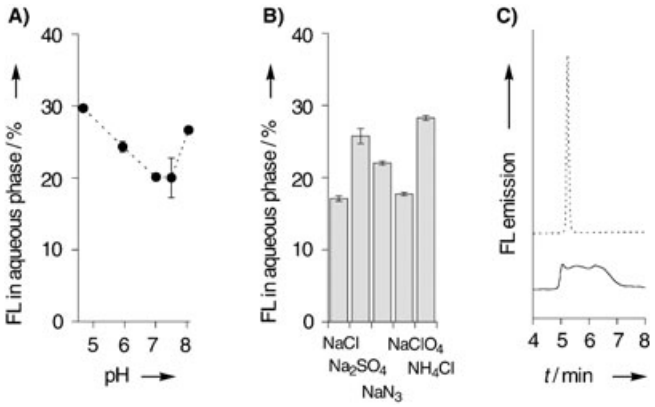

Figure 2. *Dependence of anion-mediated phase transfer of (FL-R_8)–anion complexes (10 μM) from water into chloroform containing 25 μM PG on A) pH and B) hydrophilic anions. Results are given as percentage of residual (FL-R_8)–anion complexes in the aqueous layer (10 mM $Na_mH_nPO_4$, A) 100 mM NaCl, B) pH 7.6, 100 mM NaX or 50 mM Na_2SO_4). C) HPLC of (FL-R_8)–anion complexes in buffer (top) and in bulk chloroform membranes containing PG (bottom); similar results were obtained with FL-R_{16}.*

Counteranion-mediated phase transfer of (FL-R_n)–anion complexes from water into chloroform was very fast, nearly complete within 30 min (not shown). To determine the dependence on pH and the nature of the hydrophilic anion in the aqueous phase, the concentration of PG in the organic phase was reduced until incomplete transfer was secured. This allowed for the detection of residual FL concentration in the

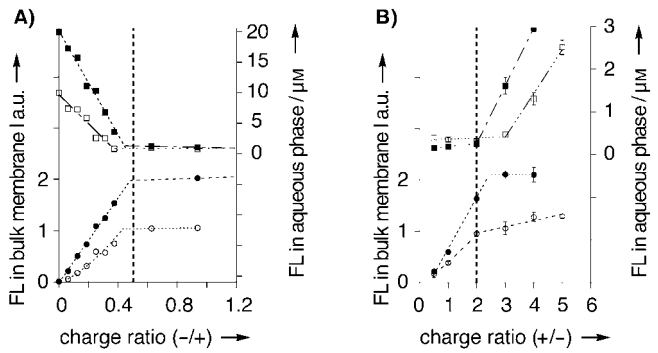

Figure 3. *Dependence of anion-mediated phase transfer of (FL-R_8)–anion complexes (filled symbols) and (FL-R_{16})–anion complexes (open symbols) from water into bulk chloroform membranes with PG on anion/cation ratio. Results are given as FL concentration or emission in aqueous (squares) and bulk membrane (circles) with A) FL-R_8 (20 μM) and FL-R_{16} (10 μM) constant, PG varied (0–150 μM) and B) FL-R_8 (1.25–10 μM) and FL-R_{16} (0.625–6.25 μM) varied, PG constant (20 μM, nearly the same with 50 μM), all in 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4.*

water. Anion-mediated phase transfer of (FL-R_n)–anion complexes was nearly independent of pH and hydrophilic anions (Figure 2 A and B). In all dilution series, transfer efficiency increased until a (FL-R_n)/PG ratio corresponding to a cation/anion ratio of ≈2 was reached (Figure 3). This finding was consistent with phase transfer of 2:1 guanidinium–phosphate complexes (Figure 1 B).[34] Stoichiometric association at various concentrations was implicative of a global dissociation constant $K_D < 10^{-5}$ M.

Counteranion-mediated phase transfer of (FL-R_n)–anion complexes from water into chloroform depended strongly on the nature of the amphiphilic counterion (Figure 4). Amphiphilic carboxylates, such as pyrenebutyrate and stearate were unable to mediate transfer of (FL-R_8)–anion complexes into bulk chloroform membranes at low (50 μM) and high concentration (1 mM, not shown). Amphiphilic phosphates and sulfates, however, mediated complete transfer of (FL-R_n)–anion complexes at low and high concentrations (PG, AOT, CS). The nearly identical results obtained with different buffers demonstrated the negligible effect of hydrophilic anions (Figure 4, filled versus open bars).

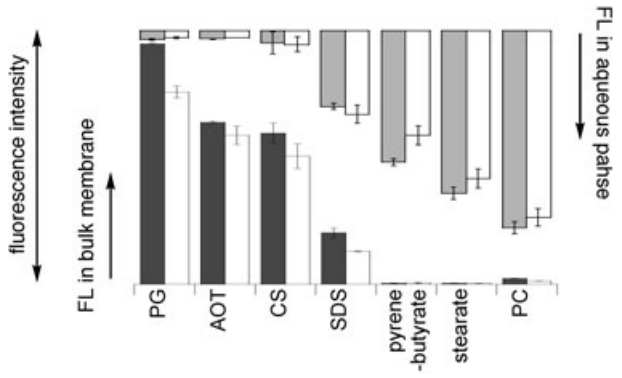

Figure 4. *Dependence of anion-mediated phase transfer of (FL-R_8)–anion complexes (10 μM) from water into bulk chloroform membranes on amphiphilic anions (50 μM PG, AOT, CS, SDS, pyrenebutyrate, stearate or zwitterionic PC). Results are given as fluorescence emission intensities of (FL-R_8)–anion complexes in aqueous (top down) and chloroform layer (bottom up; 10 mM $Na_mH_nPO_4$ (filled bars) or Tris (open bars), 100 mM NaCl, pH 7.5).*

SDS showed the highest activity at intermediate concentrations around 200 μM (Figure 5 A). The bell-shaped concentration dependence for SDS was indicative of self-assembly of these active amphiphilic anions into inactive hydrophilic polyanions (i.e., SDS micelles) at high concentration that hinder rather than mediate phase transfer of (FL-R_8)–anion complexes into bulk chloroform membranes.

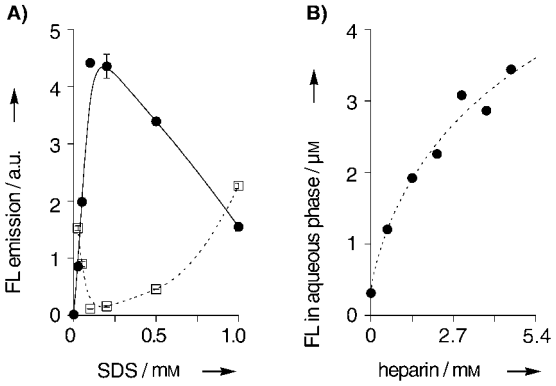

Figure 5. *Inhibition of anion-mediated phase transfer of (FL-R_8)–anion complexes (10 μM) from water into bulk chloroform membranes by hydrophilic polyanions such as SDS micelles and heparin. A) Relative emission of (FL-R_8)–anion complexes (10 μM) in the aqueous (□) and chloroform (●) layers in the presence of increasing concentrations of SDS (10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.5). B) Concentrations of (FL-R_8)–anion complexes (10 μM) in the aqueous layer after phase transfer from water (10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4) into bulk chloroform membranes (100 μM PG) with increasing heparin concentration.*

Phase transfer of (FL-R_n)–anion complexes from water into chloroform mediated by amphiphilic anion PG could be partially inhibited by heparin in the aqueous phase (Figure 5 B). However, heparin inhibition required high concentrations and did not reach completion.

Anion-mediated phase transfer of FL-oligoarginines from bulk chloroform membranes into water

Phase-transferred (FL-R_n)–anion complexes were useful for studying anion-mediated reverse transfer from bulk chloroform membranes into water. This unique opportunity to dissect translocation of (FL-R_8)–anion complexes across bulk membranes into two separate events greatly simplified the experiments and the interpretation compared to conventional "U-tube" experiments.[1,35] Moreover, the opportunity to gain specific insights into reverse transfer from bulk membranes into water was attractive because this situation might simulate intracellular release of oligoarginine carriers from biomembranes, a process probably of importance with CPPs that is difficult to separate from other processes in more complex systems.

To study reverse transfer from bulk membranes into water, nearly 10 μM of (FL-R_8)–anion complexes were dissolved in

chloroform in the presence of 100 μM PG. Then, the original aqueous layer was replaced by the aqueous solution of interest and translocation of (FL-R_8)–anion complexes from chloroform to water was determined. Reverse-phase transfer of (FL-R_8)–anion complexes increased with increasing concentration of heparin in the water phase (Figure 6A, filled circles). This finding confirmed the possibility of reverse-phase transfer of hydrophobic (FL-R_8)–anion complexes by counteranion exchange from amphiphilic PG to hydrophilic heparin. Under the same conditions, no reverse-phase transfer was found for hydrophobic (FL-R_{16})–anion complexes (Figure 6A, empty squares). This difference supported the view that the poor activity of (FL-R_{16})–anion complexes as CPPs originates from poor release from biomembranes.

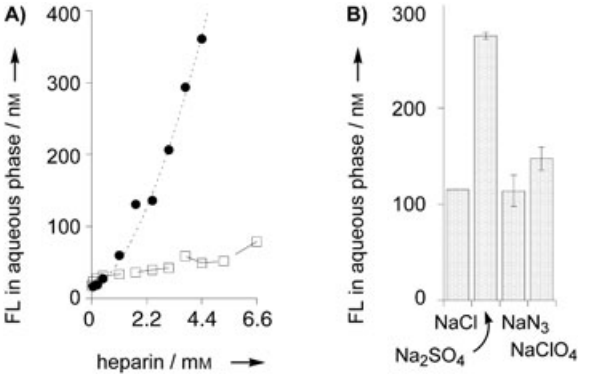

Figure 6. *Dependence of anion-mediated reverse-phase transfer of (FL-R_8)– and (FL-R_{16})–anion complexes from bulk chloroform membranes with PG (100 μM) into water on A) heparin concentration and B) the nature of the hydrophilic anions. A) 10 μM (FL-R_8)–anion complexes (●) or 10 μM (FL-R_{16})–anion complexes (□), 0–8.25 mM heparin, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4. B) 10 μM (FL-R_8)–anion complex, 10 mM $Na_mH_nPO_4$, pH 7.5 and, from left to right, 1 M NaCl, 0.5 M Na_2SO_4, 1 M NaN_3 or 1 M $NaClO_4$.*

Counteranion-mediated reverse-phase transfer of (FL-R_8)–anion complexes was not limited to hydrophilic polyanions like heparin. Consistent with the strong interactions with guanidinium cations, sulfate anions showed highest activity among the evaluated examples of small hydrophilic anions (Figure 6B).

Anion-mediated phase transfer of FL-oligoarginines from water into anionic lipid-bilayer membranes

To study the phase-transfer properties of FL-R_n, large unilamellar vesicles (LUVs) composed of PC/PG in a molar ratio of 1:1 were selected as typical model anionic bilayer membranes.[1,9,10,12,13,18] Addition of increasing concentrations of PC/PG LUVs to an aqueous solution of (FL-R_8)–anion complexes resulted in immediate quenching. The concentration dependence was indicative of tight binding of (FL-R_8)–anion complexes to anionic PG/PC LUVs (Figure 7A, filled circles).[12] However, binding to anionic LUVs did not change the emission maximum of (FL-R_8)–anion complexes at 514 nm. As red-shifted emission maxima were observed for (FL-R_8)–anion–PG complexes in nonpolar solvents (Figure 7C), this finding suggested

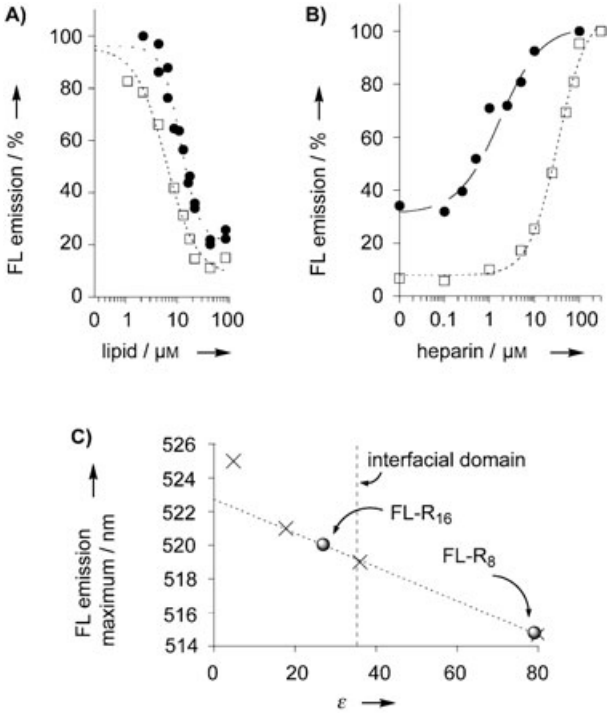

Figure 7. *Dependence of anion-mediated phase transfer of (FL-R_8)–anion complexes (A: 1 μM, B: 0.1 μM; ●) and (FL-R_{16})–anion complexes (A: 0.5 μM, B: 0.1 μM; □) from water into lipid-bilayer membranes [1:1 PG/PC-LUVs] on A) the concentration of lipids (PG + PC) and B) hydrophilic polyanions in water (heparin). C) Dependence of the emission maximum of (FL-R_8)–anion complexes on the dielectric constant ε of the environment (X = $CHCl_3$, n-butanol, methanol and water, left to right) with emission maxima of (FL-R_8)– and (FL-R_{16})–anion complexes (0.1 μM) in 1:1 PG/PC-LUVs (100 μM lipid, ●) and ε ≈ 35 of the interfacial membrane domain (dotted vertical line). Buffer: 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4.*

that (FL-R_8)–anion complexes bind to the anionic surface of 1:1 PG/PC-LUVs. This conclusion is not in agreement with the recent results by Thorén et al.; here the location of R_7W was estimated to be 10–11 Å from the center of the bilayer.[10] This difference might, however, originate from the different reporter groups used. Compared to (FL-R_8)–anion complexes, binding of (FL-R_{16})–anion complexes to anionic LUVs resulted in increased quenching (Figure 7A, empty squares) and a red shift of the FL emission maximum to 520 nm (Figure 7C). Comparison with FL emission in various nonpolar solvents implied that this red shift corresponded to a dielectric constant $\varepsilon \approx 20$ for the environment of membrane-bound (FL-R_{16})–anion complexes. This value indicated that (FL-R_{16})–anion complexes accumulated near the ester carbonyl groups between the polar head and hydrophobic core of anionic 1:1 PG/PC-bilayers.[36,37] The location of (FL-R_8)– and (FL-R_{16})–anion complexes in 1:1 PG/PC membranes was, therefore, different, the former resting at the surface and the latter between interface and hydrophobic core.

In the presence of increasing concentrations of heparin, the quenching of emission of (FL-R_8)–anion complexes upon addition of 1:1 PG/PC-LUVs decreased. This suggested that the binding of (FL-R_8)–anion complexes to 1:1 PG/PC-LUVs could be inhibited by hydrophilic polyanions. The heparin concentra-

tion for 50% inhibition of membrane binding was $IC_{50} = 0.93 \pm 0.22$ μM for (FL-R_8)–anion complexes (Figure 7B, filled circles). Confirming the stronger and "deeper" binding of (FL-R_{16})–anion complexes to 1:1 PG/PC-LUVs, a clearly higher $IC_{50} = 18.3 \pm 2.1$ μM was obtained for inhibition by heparin (Figure 7B, empty squares).

Anion-mediated reverse-phase transfer of FL-oligoarginines across intact anionic lipid-bilayer membranes into water

Overall, these findings suggested that the anion-mediated reverse-phase transfer of oligoarginines could also be studied with lipid-bilayer membranes. Specifically, anion-mediated phase transfer of FL-R_n from water into lipid bilayers would be reported as a decrease in FL emission, and anion-mediated reverse transfer of FL-R_n from lipid bilayers into water as an increase in FL emission (Figure 8). This experiment is similar to that reported by Matsuzaki et al., to monitor translocation of peptides across bilayer membranes.[38,9]

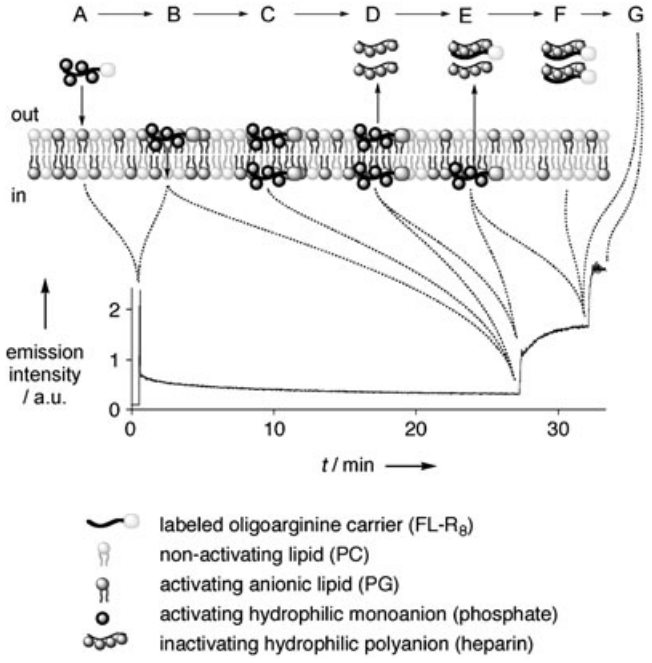

Figure 8. *Assay for direct observation of anion-mediated reverse-phase transfer of (FL-R8)–anion complexes from (and across) lipid-bilayer membranes [1:1 PG/PC-LUVs] into water (top) with representative original curve (bottom). Binding to the anionic bilayer, detected by immediate quenching (A→B), is followed by incubation for "invisible" translocation from outer to inner surface (B→C), addition of heparin (D), heparin-mediated phase transfer from outer (D→E) and inner surface (E→F), detected by biphasic dequenching, and lysis (G) for calibration. For example, changes in FL emission (a.u., arbitrary units) as a function of time during A→G for 2.1 μM 1:1 PG/PC, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.5 + (A) 0.1 μM FL-R8 + (D) 0.11 mM heparin + (G) excess triton X-100.*

To test this hypothesis, (FL-R_8)–anion complexes were added to 1:1 PG/PC-LUVs. Phase transfer into the anionic bilayers was observed as immediate quenching of the FL emission (Figure 8A→B). The resulting suspension was incubated for some minutes to allow (FL-R_8)–anion complexes to translocate across the hydrophobic core to the intravesicular bilayer surface (Figure 8B→C). Inward translocation was not visible during the incubation time; however, the feasibility of FL-R_8's moving across the hydrophobic barrier would be suggested by its ability to penetrate into bulk chloroform in the presence of PG (Figure 1): direct detection of anion-mediated CPP translocation across the membrane is made possible in this assay during reverse translocation across the membrane to a polyanion (like heparin) in the media (Figures 8E→F and 9). This reverse-phase transfer of (FL-R_8)–anion complexes from anionic bilayers into the media was initiated by the addition of heparin (Figure 8D). An immediate increase of FL emission was suggestive of phase transfer of (FL-R_8)–anion complexes located at the outer membrane surface (Figure 8D→E). The following slow increase of FL emission implied translocation of (FL-R_8)–anion complexes at the inner membrane surface to the outer surface and then phase transfer to the aqueous media (Figure 8E→F). Finally, the vesicles were lysed with triton X-100 for calibration (Figure 8F→G). The observed emission intensities at saturation (about 80 s after heparin addition) were independent of the incubation time and nearly identical to that of aqueous FL-R_8–heparin complexes, thus indicative of full re-

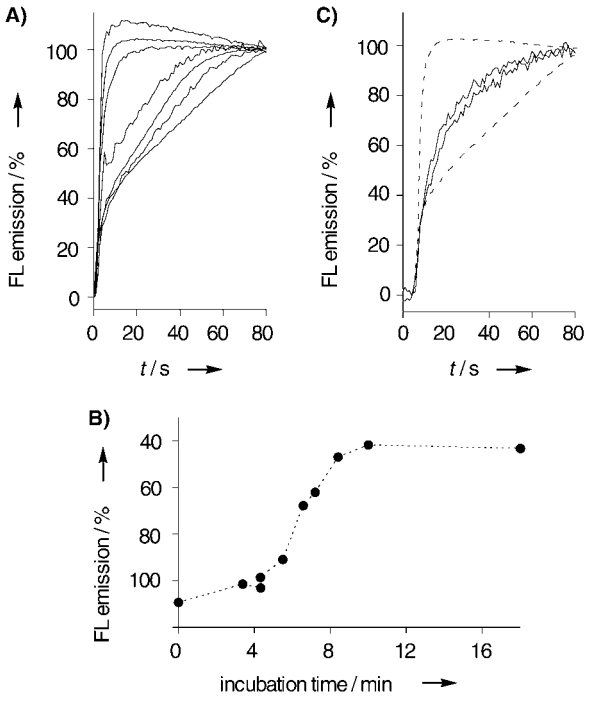

Figure 9. *Dependence of anion-mediated reverse-phase transfer of (FL-R_8)–anion complexes (A, B) and (FL-R_{16})–anion complexes (C) from and across lipid-bilayer membranes into water on incubation time. A) Increase in FL emission as a function of time after addition of (FL-R_8)–anion complexes (1 μM) to 1:1 PG/PC-LUVs (21 μM), incubation for, with decreasing emission at 10 s, 0, 3, 5, 6, 10 and 18 min, and addition of heparin at time 0 (0.11 mM) (Figure 8D→F). B) FL emission at 10 s in (A) as a function of incubation time. C) solid: as in A for (FL-R_{16})–anion complexes (0.1 μM) in 1:1 PG/PC-LUVs (2.1 μM), with incubation times of 1 and 25 min; (dashed): (FL-R_8)–anion complexes with comparable incubation time (from A).*

lease of FL-R_8 from the bilayer membrane. The increase in emission upon addition of Triton X-100 originates from interactions between aqueous FL-R_8–heparin complexes and (mixed) triton micelles (rather than the release of residual bound peptide).

The biphasic behavior found for heparin-mediated reverse-phase transfer suggested that 1) (FL-R_8)–anion complexes translocate across the hydrophobic core of bilayer membranes from one surface to the other, 2) the bilayer remains intact during this process (Figure 8D→F), and 3) direct detection of this process by fluorescence kinetics is possible. These results supported the notion of (FL-R_8)–anion complexes acting as carriers under these conditions, mediating, for example, the previously reported efflux of entrapped hydrophilic reporter anions like carboxyfluorescein.[1] The direct observation of anion-mediated reverse-phase transfer across intact bilayers (Figure 8E) is particularly attractive because it simulates the movement of CPPs from a cell surface across the membrane to a hydrophilic polyanion in the cytoplasm, for example, RNAs and ATP (Figure 8E).

The significance of these interpretations called for several control experiments. The above results in bulk chloroform membranes provided evidence for the feasibility of anion-mediated phase transfer of (FL-R_8)–anion complexes into and across hydrophobic domains (compare, e.g., Figure 1). Moreover, according to the FL-emission maximum, the accumulation of (FL-R_8)–anion complexes at the surface of 1:1 PG/PC-LUVs did not change with incubation time (Figure 7C). Considering that the possible translocation step of (FL-R_8)–anion complexes from one surface across the hydrophobic core to the other bilayer membrane surface was slow (Figure 8E→F), it was conceivable that the kinetics of reverse-phase transfer would not be biphasic after only short incubation (Figure 8B→C). This was found to be true (Figure 9A). Incubations shorter than five minutes resulted in complete removal of all (FL-R_8)–anion complexes immediately after heparin addition, whereas unchanged biphasic kinetics were observed after incubation beyond 10 min (Figure 9A). The resulting sigmoidal kinetics suggested that anion-mediated translocation of (FL-R_8)–anion complexes across the hydrophobic core of bilayer membranes is an autocatalytic process (Figure 9B). Sigmoidal kinetics were observed previously on the same timescale for the transport of CF reporter anions by polyarginine–anion complexes across PG/PC membranes.[1]

Anion-mediated translocation of (FL-R_8)–anion complexes across the hydrophobic core of bilayer membranes further depended on the lipid/CPP ratio as expected. Specifically, biphasic kinetics for heparin-mediated phase transfer of (FL-R_8)–anion complexes from bilayer membranes into water disappeared with increasing 1:1 PG/PC-LUV concentration at constant incubation time and CPP concentration (not shown). These results were consistent with an earlier failure to detect the entry of arginine-rich peptides into LUVs under comparable conditions.[9,10] In clear contrast to the situation with (FL-R_8)–anion complexes, the continuous and slow reverse-phase transfer of (FL-R_{16})–anion complexes from bilayer membranes into water did not significantly depend on the incubation time before heparin addition (Figure 9C). This behavior was suggestive of strong and "deep" binding of FL-R_{16} near the hydrophobic core of the bilayer.

The considerable involvement of endocytosis in the cellular uptake in intracellular delivery with CPPs has recently been pointed out.[7,23] However, the delivered molecules cannot be bioactive without entering the cytosol, and there are reports on the cellular uptake of CPPs that cannot be explained by endocytosis.[24,25,27] It is therefore critical to clarify the precise mechanisms of translocation in order to establish more sophisticated delivery systems into cells. The idea of possible contributions from counteranions to the membrane-permeation step described in this report might provide an important clue to the understanding of their translocation mechanisms. The interaction of HIV-1 Tat peptide, one of the representative arginine-rich CPPs, with extracellular glycosaminoglycans and its possible relevance to the internalization mechanisms has been suggested previously. The proposed mechanisms, however, mainly focused on the involvement of cell-surface adsorption and the concentration of the peptides at the initial stage of internalization.[12,16,21,22,28,29] The concept of counterion-mediated dynamic inversion of charge and solubility[1] elaborated in this report considers counteranion control throughout the entire translocation process of CPP with particular emphasis on the possible contributions of cytosolic anions, such as RNA and ATP to the release of arginine-rich CPPs from the membrane.

Octaarginine (R_8) is one of the most efficient CPPs in HeLa, RAW264.7, and COS-7 cells.[8] In the earlier study with the fixed cells, the cellular uptake of R_8 peptide was regarded to be higher than that of the arginine 16-mer (R_{16}). However, it was recently pointed out that significant artifacts could be caused by strong adhesion of the basic peptides to the cell surfaces followed by fixation of the cells for microscopic observation.[23] Re-examination with live cells, which took the above problem into consideration, suggested that, although the total cellular uptake of R_{16} was higher than that of R_8, the amount of cytosol-released peptides for both peptides was comparable (unpublished data). Zaro and Shen also suggested similar results using arginine 9-mers and 15-mers.[19] These results suggested that R_{16} translocates through biological membranes less efficiently than R_8 due to its stronger adhesion to the membranes. The findings reported here on the differences between R_8 and R_{16} in model membranes are in excellent agreement with these insights from live cells.

Conclusion

Dissection of complex phase-transfer processes to detect individual unidirectional translocations proved crucial to obtaining more informative insights into the consequences of anion-mediated dynamic inversion of solubility and the charge of (FL-R_n)–anion complexes on function. Little difference could be observed for anion-mediated phase transfer of (FL-R_8)– and (FL-R_{16})–anion complexes into bulk and bilayer membranes. Amphiphilic counteranions, like PG, AOT, CS or SDS, mediated full transfer of both polycations into chloroform and PG/PC membranes, whereas hydrophilic counteranions, like heparin

or sulfate, served as general inhibitors. FL-R_{16} accumulated between interfacial and hydrophobic lipid bilayer domains, whereas FL-R_8 remained at the membrane surface. Clear differences were, however, observed between (FL-R_8)– and (FL-R_{16})–anion complexes for anion-mediated reverse-phase transfer from bulk and lipidic membranes into the water phase. (FL-R_{16}) complexed with amphiphilic anions (PG) was difficult to ion-exchange with hydrophilic polyanions (heparin), whereas heparin-mediated reverse-phase transfer of (FL-R_8)–anion complexes was unproblematic. Translocation of (FL-R_8)–anion complexes from outer and inner bilayer surfaces to polyanions in the water phase could be differentiated. This finding provided direct access to detect the translocation of (FL-R_8)–anion complexes across the hydrophobic core of intact lipid-bilayer membranes. Consistent with previous findings,[1] FL-R_8-carriers turned out to act, under the given conditions, within minutes in an autocatalytic manner.

In summary, it was possible to simulate, isolate and characterize each phase-transfer process required for cell penetration of FL-R_8-carriers across biomembranes. The differences found for phase transfers of (FL-R_{16})– and (FL-R_8)–anion complexes further matched the differences in their manners of membrane interaction and translocation. It is evident that counteranion-mediated dynamic inversion of charge and solubility as such will not explain all the functions of oligoarginines in biomembranes.[5–32] However, the reported results do underscore the importance of counteranions. In other words, the "mysterious" functions of oligoarginines in biomembranes cannot be understood if the dynamic (i.e., elusive) contributions from the rich cocktail of extra- and intracellular counteranions as well as those in the biomembranes offered by live cells are ignored.

Experimental Section

Materials. Heparin (sodium salt, 17–19 kDa), sodium dodecylsulfate (SDS), bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT), Triton X-100, buffers and salts were purchased from Sigma, stearic acid was purchased from Fluka, 1-pyrenebutyric acid was purchased from Acros Organics, egg yolk phosphatidylglycerol (PG) and cholesterol sulfate (CS) were from Northern Lipids, and egg yolk phosphatidylcholine (PC) was from Avanti Polar Lipids. Vesicles were prepared by using a Mini Extruder with a polycarbonate membrane (Avanti Polar Lipids, pore size 100 nm). Fluorescence measurements were performed either on a Fluoromax 2 or on a Fluoromax 3 (Jobin-Yvon Spex) spectrometer, both equipped with a stirrer and a temperature controller (25 °C). HPLC was performed by using a Jasco HPLC system with a fluorescence detector (FP-920). UV spectra were measured by using a Varian Cary 100 Bio UV/vis spectrometer.

Synthesis of FL-R_8 and FL-R_{16}. FL-labelled oligomers were prepared by Fmoc solid-phase peptide synthesis on a Rink amide resin where a χ-aminobutyryl residue was employed as a linker connecting the N-terminal FITC moiety to the arginine peptides. The FITC-labelled peptide resin was treated with trifluoroacetic acid (TFA)/ethanedithiol (95:5). The peptides were purified by RP-HPLC, and the molecular masses of the peptides were confirmed by MALDI-TOF MS. Concentrations of aqueous stock solutions were confirmed by UV/vis spectroscopy.

Phase transfer from water into bulk chloroform membranes (general procedure A). A solution of PG (0.1 mM) in $CHCl_3$ (0.2 mL) and an aqueous solution (0.18 mL, 11.1 mM $Na_mH_nPO_4$, 111 mM NaCl, pH 7.4) were placed in a vial and mixed vigorously. FL-R_8 (20 μL of 0.1 mM aqueous solution) was added to this biphasic solution, (10 μM final concentration in aqueous layer). Then, the biphasic solution was vortexed and placed on a shaker (200 rpm) for >30 min at 37 °C. Kinetics measurements confirmed that phase transfer was nearly complete within 30 min. Then, 20 μL of the $CHCl_3$ layer was added to MeOH (1.48 mL), and the FL emission intensity was determined ($\lambda_{ex}=495$ nm, $\lambda_{em}=519$ nm). Fluorescence emission intensity thus obtained could be used to estimate the concentration of oligopeptides in the organic layer; however, fluorescence of aqueous layer was more easily reproducible and reliable. Thus, 20 μL of the aqueous layer was added to the buffer (1.48 mL, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4), and the FL-R_8 concentration was determined from the FL emission intensity ($\lambda_{ex}=495$ nm, $\lambda_{em}=517$ nm) by using calibration curves for these conditions. All experiments were performed at least twice, and average values ± errors were reported. All data points in one graph are from the parallel experiments.

(Figure 1C): According to general procedure A, FL-R_8 (25 μM) in aqueous buffer (0.4 mL, 10 mM $Na_mH_nPO_4$, pH 7.4, 100 mM NaCl) was incubated with the $CHCl_3$ layer (0.4 mL) with or without PG (0.25 mM) at 37 °C for 30 min.

RP-HPLC analysis (Figure 2C). 1 μL of an aqueous solution of FL-R_8 (1 mM) and FL-R_{16} (1 mM) was subjected to HPLC under the following conditions: column, Agilent Eclipse XDB-C8 4.6×150 mm, mobile phase, linear solvent gradient from water/TFA 99:1 to CH_3CN, over 10 min, flow rate 1 mL min^{-1}, fluorescence detection ($\lambda_{ex}=495$ nm, $\lambda_{em}=517$ nm). Retention times were $R_t=5.24$ min for FL-R_8 and $R_t=5.26$ min for FL-R_{16}, appearing both as sharp peaks. Then, FL-R_8 (25 or 50 μM) and FL-R_{16} (25 or 50 μM) were phase transferred into $CHCl_3$ according to general procedure A, with PG (1 mM). The HPLC chromatograms of 20 μL of the resulting aqueous layers showed very weak peaks only, whereas that of 10 μL of the resulting chloroform layers showed broad peaks $R_t=4.8$–7.5 min.

pH dependence (Figure 2A). Following general procedure A, vials containing FL-R_8 (10 μM) in aqueous buffer (0.2 mL, 10 mM $Na_mH_nPO_4$, pH 4.7–8.1, 100 mM NaCl) and PG (25 μM in $CHCl_3$, 0.2 mL) were prepared and incubated for 1 h. The concentration of FL-R_8 in the aqueous layer was determined as described in the general procedure.

Hydrophilic anions (Figure 2B). Experiments were performed according to general procedure A by using the indicated salts in place of NaCl. Namely, the aqueous layers (0.2 mL) on top of $CHCl_3$ (0.2 mL, 25 μM PG) consisted of 10 μM FL-R_8, 10 mM $Na_mH_nPO_4$, pH 7.4, and 100 mM NaCl, NaN_3, $NaClO_4$, NH_4Cl or 50 mM Na_2SO_4.

Dependence on oligopeptide and PG concentration (Figure 3)

Fixed peptide concentration (Figure 3A): The aqueous layer consisted of FL-R_8 (20 μM) or FL-R_{16} (10 μM) in phosphate buffer (10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4) and concentration of PG in organic layer was varied between 0–0.15 mM.

Fixed PG concentration (Figure 3B): The aqueous buffer (10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4) contained FL-R_8 (1.25–10 μM) or FL-R_{16} (0.625–6.25 μM) on top of the $CHCl_3$ layer containing PG (20 μM). Incubations and analyses were as in general procedure A.

Amphiphilic anions (Figure 4)

PG, PC, AOT, CS, stearate. Experiments were performed by following general procedure A and using 50 μM of PG, PC, AOT, CS or stearate in $CHCl_3$ (0.2 mL) below buffer (0.2 mL; 10 μM FL-R_8, 10 mM tris(hydroxymethyl)aminomethane (Tris) or $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4).

SDS, pyrenebutyrate: SDS, or pyrenebutyrate (50 μM) was included in the aqueous layer (0.2 mL; 10 mM, $Na_mH_nPO_4$, or Tris, pH 7.4, 100 mM NaCl, 10 μM FL-R_8) on top of $CHCl_3$ (0.2 mL). Following general procedure A, the biphasic mixtures were vortexed before and after the addition of FL-R_8.

Hydrophilic polyanions

SDS concentration dependence (Figure 5A). SDS (0.025–1 mM) was included in the buffer (0.2 mL; 10 μM FL-R_8, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4) on top of $CHCl_3$ (0.2 mL). The biphasic mixtures were vortexed before and after the addition of FL-R_8.

Heparin concentration dependence (Figure 5B). Heparin (0–4.7 mM) was included in the aqueous layer (0.2 mL; 10 mM, $Na_mH_nPO_4$, pH 7.4, 100 mM NaCl, 10 μM FL-R_8) on top of the $CHCl_3$ layer (0.2 mL, 0.1 mM PG).

Phase transfer from bulk chloroform membranes into water (general procedure B). Following general procedure A, four vials containing the aqueous layer (1 mL, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4, 10 μM FL-R_8 or 5 μM FL-R_{16}) and the $CHCl_3$ layer (1 mL, 0.1 mM PG) were shaken for 2 h at 37 °C. The contents of four vials were mixed and clearly separated into aqueous and organic layers in a centrifuge. Figure 6A: aliquots (0.2 mL) of the organic layer containing FL-R_8 or FL-R_{16} complexed with PG were placed in vials containing aqueous buffer (0.2 mL, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4, heparin 0–8.25 mM). The mixtures were incubated for 2 h at 37 °C, and the amount of oligopeptide in the aqueous layer was determined as described in general procedure A. Figure 6B: experiments were performed as for Figure 6A, but with only FL-R_8 and the following buffer for re-extraction: $Na_mH_nPO_4$ (10 mM), NaCl, NaN_3, $NaClO_4$ (1 M), or Na_2SO_4 (0.5 M), pH 7.4.

Preparation of 1:1 PG/PC-LUVs. Unilamellar vesicles (LUVs) composed of equimolar PG and PC were prepared by using the extrusion method following previously reported procedures.[1] Solutions of PC and PG (25 mg, 1:1 molar ratio) in $CHCl_3$/MeOH (1:1) were dried under a stream of N_2 and then in vacuo (>2 h) to form thin films. The resulting films were hydrated with buffer (10 mM $Na_mH_nPO_4$ or *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES; for phosphate analysis), 100 mM NaCl, pH 7.4) for >30 min, freeze-thawed (5×), extruded through a polycarbonate membrane (100 nm, >15×), purified on a Sephadex G-50 column, and diluted to 3.8 mL. Phosphate analysis[33] of the resulting 1:1 PG/PC-LUV suspensions prepared in HEPES buffer gave lipid concentrations of 4.4±0.1 mM. The lipid concentration of LUVs suspension prepared with phosphate buffer was assumed to be same.

Phase transfer from water into lipid-bilayer membranes: Lipid concentration dependence (Figure 7A, general procedure C): 1:1 PG/PC-LUVs suspension (0–40 μL), prepared as described above with HEPES buffer, was placed in a cuvette and diluted with a buffer (10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4) to 2 mL. Then FL-R_8 (20 μL, 0.1 mM, final concentration 1 μM) or FL-R_{16} (20 μL, 50 μM, final concentration 0.5 μM) was added. Fluorescence emission intensities were recorded as a function of time at λ_{ex}=495 nm, λ_{em}=517 nm. Emission intensities 50 s after the addition of the peptides relative to that obtained in the absence of lipid were plotted as a function of lipid concentration.

Heparin concentration dependence (Figure 7B): PG/PC LUVs (20 μL, prepared in Na phosphate buffer) were mixed with a buffer (1.98 mL, 10 mM $Na_mH_nPO_4$, 100 mM NaCl, pH 7.4) containing varying concentration of heparin (0–55 μM), and FL-R_8 (20 μL, 10 μM, final concentration 0.1 μM) or FL-R_{16} (20 μL, 10 μM, final concentration 0.1 μM) was added to the solution. Data were obtained following general procedure C, and plotted as a function of heparin concentration.

Data analysis: IC_{50} and Hill coefficients were calculated by fitting the data to Equation (1):

$$I = I_0 + \frac{I_\infty - I_0}{1 + (IC_{50}/c)^n} \quad (1)$$

here I is the relative emission intensity, I_0 the initial value, I_∞ the value at saturation, c the concentration of the analyte, and n the Hill coefficient. Analyses were performed with KaleidaGraph, version 3.5 (Synergy Software).

Solvochromicity of FL (Figure 7C): Following the general procedure A, FL-R_8 (10 μM) was extracted into the $CHCl_3$ layer (1 mL, containing 0.1 mM PG) from aqueous buffer (1 mL, 10 mM $Na_mH_nPO_4$, pH 7.4, 100 mM NaCl). An aliquot (10 μL) of the resulting $CHCl_3$ solution was diluted with $CHCl_3$, *n*BuOH, or MeOH (1.5 mL), and emission spectra of the solution were taken (λ_{ex} at 495 nm). Emission spectra in aqueous system were obtained with FL-R_8 or FL-R_{16} (67 nM) in a buffer (1.5 mL, 10 mM $Na_mH_nPO_4$, pH 7.4, 100 mM NaCl) with or without PG/PC LUVs (50 μM). Identical emission maxima were observed with a higher peptide-to-lipid ratio (FL-R_8 1 μM, PG/PC LUVs 22 μM) and after 10 min of incubation.

Reverse phase transfer (general procedure D, Figure 8): FL-R_8 (0.1 μM) was added to a dispersion of PG/PC LUVs (2.1 μM, freshly prepared in phosphate buffer) in a buffer (2 mL, 10 mM $Na_mH_nPO_4$, pH 7.4, 100 mM NaCl). After 27 min, heparin (40 μL of 5.5 mM) was added, followed by triton X-100 (0.024%). Fluorescence emission intensities were recorded during the entire processes at λ_{em}= 517 nm and λ_{ex}=495 nm. Note, use of fresh buffers, fresh vesicles, and plastic cuvettes was crucial to obtain reproducible results. Control experiments with a glass cuvette in the absence of LUVs resulted in increased fluorescence upon addition of heparin, while with plastic cuvette no change was observed. These results are in agreement with the adsorption of oligoarginine on the electronically negative surface of glass cuvette, as was observed with penetration.[18]

Figure 9A and B: By following general procedure D, FL-R_8 (1 μM) was incubated for given time with PG/PC LUVs (21 μM) in a buffer (2 mL, 10 mM $Na_mH_nPO_4$, pH 7.4, 100 mM NaCl). The fluorescence intensity after the addition of heparin was normalized from 0 (before addition of heparin) to 100% (80 s after addition of heparin), and the percentage emission intensity at 10 s after addition of heparin was plotted as a function of incubation time to give Figure 9B.

Figure 9C: Experiments were performed as described in general procedure D, but with FL-R_{16} (0.1 μM).

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