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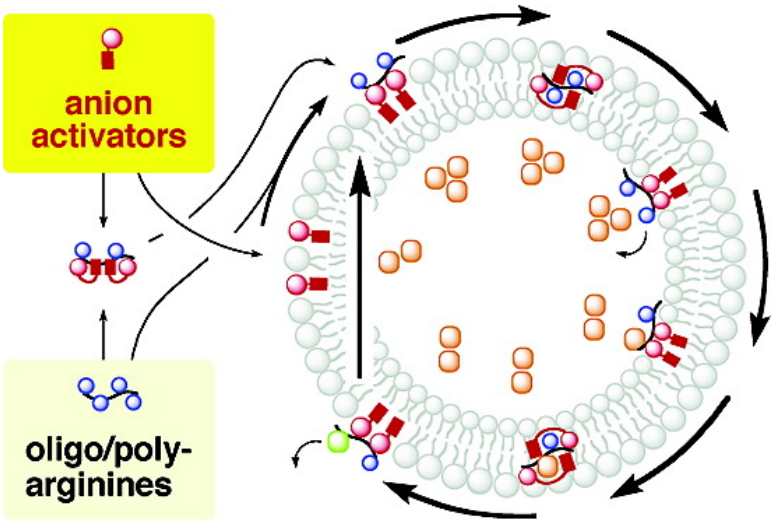

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Anionic Fullerenes, Calixarenes, Coronenes, and Pyrenes as Activators of Oligo/Polyarginines in Model Membranes and Live Cells

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Cell-penetrating peptides (CPPs) are of widespread interest as potential drug delivery agents.[1−3] Arginine oligomers are among the simplest of the CPPs. We have recently shown that the identity of the counteranions can have a large effect on CPP behavior in lipid bilayer vesicles.[1,2] Herein we show that counteranions with large aromatic groups such as **1**−**8** (Figure 1) are strong enablers of CPP activity and that these effects are seen with live cells as well as in vesicle model systems.

The carrier activity of oligo/polyarginines in anionic vesicles containing egg yolk phosphatidylglycerol (EYPG) is thought to occur by counterion exchange to reversibly adapt their solubility to changing environments.[1−4] The question thus arose whether the EYPG anion could be replaced by amphiphilic anions added to the media to activate oligo/polyarginine carriers in otherwise inaccessible egg yolk phosphatidylcholine (EYPC) vesicles. To exemplify this new expression of counteranion-mediated function, pyrenebutyrate **5** was selected as representative activator **Y** (Figure 1). Poly-L-arginine (pR)-anion (X) complexes pR•$(X)_m$ were chosen as carriers[1−4] $\mathbf{R_n}$•$(X)_m$ and EYPC-LUVs⊃CF as model membranes to detect carrier activation by the dequenching of entrapped CF during release (EYPC-LUVs⊃CF: Large unilamellar vesicles composed of EYPC and loaded with 5(6)-carboxyfluorescein).[1,2] According to constant CF emission (Figure 2B, a → b), partial[5] partitioning of **5** to EYPC bilayer did not cause CF release under the micromolar concentrations of interest (Figure 1a′). Upon addition of pR•$(X)_m$ complexes, anion exchange either in the media (a) or at the membrane−water interface (b) was expected to yield the desired, anion-activated pR•$(X)_{m'}$•$(\mathbf{5})_{m''}$ carriers at the outer membrane surface (Figure 1B). Phase transfer (C), intravesicular anion exchange (D, E), back-transfer (F), and extravesicular anion exchange (G) would then result in the release of readily detectable, dequenched CF into the media.

Increasing CF release found with increasing concentration of activator **5** at constant pR•$(X)_m$ concentration was in support of these expectations (Figure 2B, b → c). Hill analysis of the obtained curves gave Y_{max} (i.e., maximal CF release relative to lysis) and EC_{50} (i.e., the effective activator concentration needed to reach $Y_{max}/2$) as characteristics of activator **5** (Figure 2A).[6,7] Variation of spacer length and the nature of the anions gave decreasing Y_{max} with decreasing EC_{50} for the pyrene series **4**−**8** (Figure 2A, filled circles, dotted line).[8] Hexa-L-arginine carriers (R_6) gave similar results at the correspondingly higher concentrations.

The roughly exponential EC_{50}−Y_{max} profile for the pyrene series identified activators **Y** with intermediate affinity and hydrophobicity as ideal. This finding was frustrating from a practical point of view

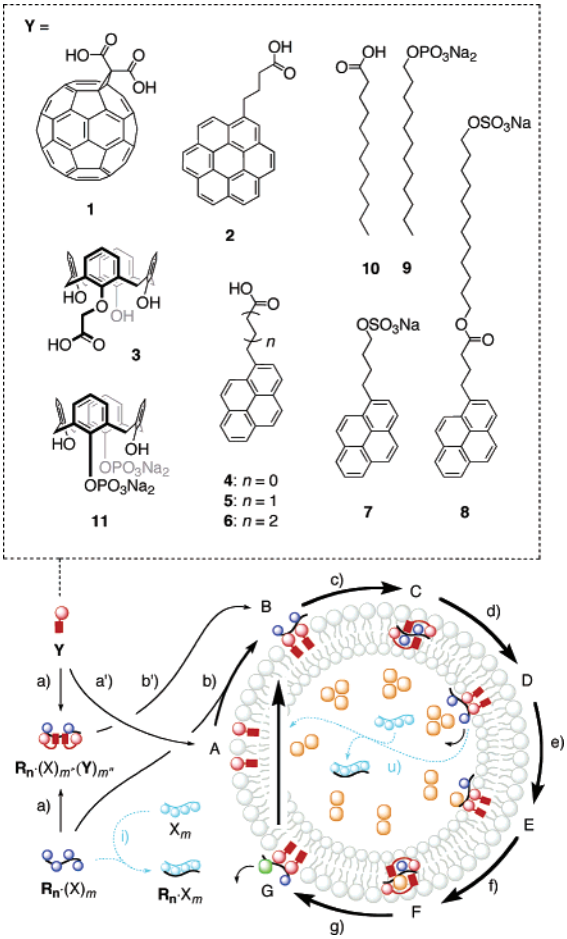

Figure 1. Concept of counteranion-mediated function of oligo/polyarginines $\mathbf{R_n}$ (black) in EYPC-LUVs⊃CF (gray) with structure of anion activators **Y** (red): Binding of complexes $\mathbf{R_n}$•$(X)_m$ to (a) aqueous or (b) interfacial **Y** produces complexes $\mathbf{R_n}$•$(X)_{m'}$•$(\mathbf{Y})_{m''}$ (B) that shuttle across the bilayer (c, d), pick up entrapped, self-quenched CF (e, orange), and back-transfer (f, g) to release unquenched CF (green, G → B). X_m = hydrophilic polyanions such as glycosaminoglycans, RNA, or SDS micelles as (i) inhibitors or (u) uptake mediators (cyan).[1,2] X = scavenged hydrophilic anions such as inorganic phosphate (blue).

because it implied that counteranion-mediated oligo/polyarginine activation would be efficient only with mediocre anions at high anion concentrations. These concerns were supported by the failure to reach beyond the "pyrene threshold" (Figure 2A, dotted line) using high-affinity anions such as sulfates and phosphates with various hydrophobic tails (e.g., **9**).[9] The stunning difference in Y_{max} between alkyl (**10**) and pyrene (**5**) carboxylates with nearly identical EC_{50}, however, revealed higher aromatics as promising activators

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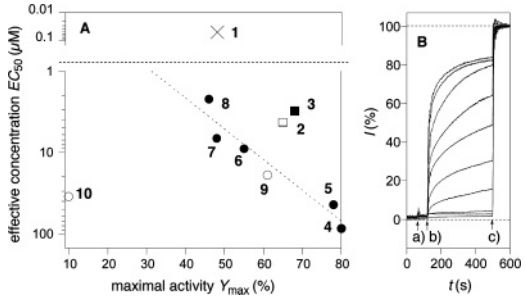

Figure 2. (A) Effective concentrations of anion activators **Y** (Figure 1) and maximal activity of anion−polyarginine complexes to mediate CF release from EYPC-LUVs⊃CF (dotted line: exponential curve fit for pyrene series, ●). (B) Original data for activation of poly-L-arginine in EYPC-LUVs by pyrenebutyrate **5**: Change in CF emission I (λ_{ex} 492 nm, λ_{em} 517 nm) as a function of time during the addition of activator **5** (a, with increasing activity b → c, 0, 1, 5, 10, 20, 30, 40, 50, 75, and 100 μM) and pR (b, 250 nM) to EYPC-LUVs⊃CF (13 μM EYPC), calibrated by final lysis (c, excess Triton X-100).

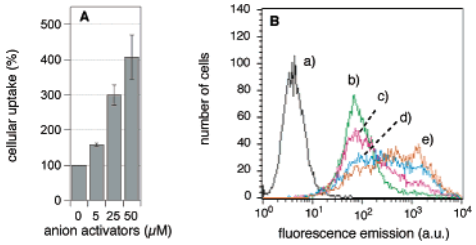

Figure 3. (A) Uptake of R_8-alexa by HeLa cells with increasing concentration of activator **5**. (B) Original flow cytometry data. Fluorescence histogram for HeLa cells after incubation with pyrenebutyrate **5**: 0 (a, b), 5 (c), 25 (d) and 50 μM (e) and R_8-alexa: 0 (a) and 5 μM (b−e) in arbitrary units (a.u.).

that may (a) mediate interface-directed translocation[10] and (b) maximize arene-templated carboxylate−guanidinium pairing.[11] Indeed, coronenebutyrate[12] **2** gave higher Y_{max} at lower EC_{50} compared to the pyrene series. Similarly high efficiency was obtained for calix[4]arene carboxylate **3**.[13,14] As far as phosphates are concerned, the suspicion that increase in oligoargininophilicity would result in poor phosphate−guanidinium dissociation was confirmed by the change from satisfactory activity with alkyl phosphates (**9**) to inactivity with calix[4]arene diphosphate **11** (Y_{max} = 0). Corroborating the spectacular characteristics of certain fullerenes in biological systems[15] and the potential of arene-templated ion pairing,[11] fullerene malonate **1** showed an extraordinary EC_{50} with intermediate Y_{max}.

The relevance of anion activation of oligo/polyarginines to penetrate live cells was probed by incubating HeLa cells with pyrenebutyrate **5** as representative activator before addition of a fluorescently labeled octa-L-arginine, that is, R_8-alexa.[6] The cell surface-adsorbed peptides were removed by trypsin treatment of the cells prior to analysis. Gradually increasing uptake of R_8-alexa with increasing concentration of activator **5** was found by flow cytometry (Figure 3). As expected from above model studies, the dependence of uptake on the concentration of the homologous pyrene sulfate **7** was less impressive (not shown).

Taken together, these results provide experimental evidence that amphiphilic anions can be used to activate oligo/polyarginines in EYPC membranes and HeLa cells. Among a rich collection of identified activators, amphiphilic fullerene ≫ calix[4]arene ≈ coronene > pyrene carboxylates show particularly promising synergism, presumably due to favorable activator−membrane (interface-directed translocation)[10] and activator−carrier interactions (arene-templated carboxylate−guanidinium pairing).[11] Ongoing studies in live cells explore possibilities such as counteranion-mediated intra- and intercellular targeting, and work in model lipid bilayer and bulk membranes focuses on sensing applications and phase-transfer catalysis and templation in organic synthesis, respectively.

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

References

(1) Sakai, N.; Matile, S. *J. Am. Chem. Soc.* **2003**, *125*, 14348−14356.
(2) Sakai, N.; Takeuchi, T.; Futaki, S.; Matile, S. *ChemBioChem*, in press, and references therein.
(3) Partitioning of octaarginine (R_8) into octanol using laurate as a counteranion has recently been reported: (a) Rothbard, J. B.; Jessop, T. C.; Lewis, R. S.; Murray, B. A.; Wender, P. A. *J. Am. Chem. Soc.* **2004**, *126*, 9506−9507. Compare also: (b) Dom, G.; Shaw-Jackson, C.; Matis, C.; Bouffioux, O.; Picard, J. J.; Prochiantz, A.; Mingeot-Leclercq, M. P.; Brasseur, R.; Rezsohazy, R. *Nucleic Acids Res.* **2003**, *31*, 556−561.
(4) Evidence for other uptake mechanisms in live cells exists.[1,2] The existence of anion transport mechanisms other than a carrier mechanism[1,2] cannot be excluded in vesicles either, although activity in DPPC vesicles below phase transition was poor and neither ion-channel formation nor membrane disruption could be observed in planar EYPC/EYPG-bilayer experiments.
(5) (a) Jones, O. T.; Lee, A. G. *Biochemistry* **1985**, *24*, 2195−2202. (b) Martins, J.; Melo, E. *Biophys. J.* **2001**, *80*, 832−840.
(6) Please see the Supporting Information.
(7) Y_{max} depended on nature as well as concentration of oligo/polyarginine (not shown); the possibility of systematic batch-to-batch errors required attention to eventually necessary calibration.
(8) Pyrenes with low EC_{50} showed more intense excimer emission.[1]
(9) Inactivity of alcohols corresponding to all sulfates and some carboxylates listed in Figure 1 confirmed, however, that low-affinity ion pairing is essential.
(10) (a) Schiffer, M.; Chang, C. H.; Stevens, F. J. *Protein Eng.* **1992**, *5*, 213−214. (b) Yau, W. M.; Wimley, W. C.; Gawrisch, K.; White, S. H. *Biochemistry* **1998**, *37*, 14713−14718.
(11) (a) Thompson, S. E.; Smithrud, D. B. *J. Am. Chem. Soc.* **2002**, *124*, 442−449. (b) Orner, B. P.; Salvatella, X.; Sánchez-Quesada, J.; de Mendoza, J.; Giralt, E.; Hamilton, A. D. *Angew. Chem., Int. Ed.* **2002**, *41*, 117−119.
(12) Davenport, L.; Shen, B.; Joseph, T. W.; Straher, M. P. *Chem. Phys. Lipids* **2001**, *109*, 145−156.
(13) Da Silva, E.; Lazar, A. N.; Coleman, A. W. *J. Drug Del. Sci. Technol.* **2004**, *14*, 3−20.
(14) Whereas ring expansion to calix[6]arenes reduced activator efficiency, additional carboxylates on one face and alkyls on the other face of calix[4]arenes were tolerated. Several calix[4]arene carboxylates with sulfates at the upper rim were inactive, presumably because the resulting complexes were too hydrophilic.
(15) (a) Bingel, C. *Chem. Ber.* **1993**, *126*, 1957−1959. (b) Nakamura, E.; Isobe, H. *Acc. Chem. Res.* **2003**, *36*, 807−815. (c) Foley, S.; Crowley, C.; Smaihi, M.; Bonfils, C.; Erlanger, B. F.; Seta, P.; Larroque, C. *Biochem. Biophys. Res. Commun.* **2002**, *294*, 116−119.

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