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Amplifier-Mediated Activation of Cell-Penetrating Peptides with Steroids: Multifunctional Anion Transporters for Fluorogenic Cholesterol Sensing in Eggs and Blood**

*Sara M. Butterfield, Tomohiro Miyatake, and Stefan Matile**

Cell-penetrating peptides (CPPs), such as HIV-1 Tat, polyarginine (pR), and a rich collection of synthetic mimics, are attracting increasing attention owing to their usefulness in drug delivery,[1] and, more recently, as optical transducers of reactions.[2] We previously demonstrated that certain amphiphilic anions can activate CPPs to move across bulk, lipid-bilayer as well as live-cell membranes.[2,3] The concept of CPP activators builds on the fact that arginine-rich polycations permanently coexist with readily exchangeable counteranions to minimize intramolecular charge repulsion between the weakly acidic guanidinium cations.[3] Exchange of the hydrophilic counterions of CPPs in water by amphiphilic anion activators affords hydrophobic CPP–activator complexes that can move across the membrane.[3] A two-step counterion exchange from hydrophilic to amphiphilic and back to hydrophilic anions is sufficient for CPPs to overcome the membrane barrier and enter cells.[3] Activated CPP–counterion complexes can bind small hydrophilic anions such as 5(6)-carboxyfluorescein (CF) by partial counterion exchange and transport them across bulk and lipid bilayer membranes (Figure 1), although other mechanisms might also contribute to anion export from lipid bilayer vesicles.[2,3]

Owing to their commercial availability, CPP–activator complexes are attractive for sensing applications. However, to date they have proven to be inferior to synthetic pores[4–6] in addressing critical issues such as the discrimination of adenosine tri- and diphosphate (ATP and ADP)[2] or of phytate and IP_7 (diphosphoinositol pentakisphosphate).[5] For sensing with pores, enzymes have been introduced to react selectively with the analyte of interest, thus generating an analyte-specific signal.[4] Multianalyte sensing in complex matrices became possible with the introduction of signal amplifiers.[6] Signal amplifiers are bifunctional molecules that can react in situ with the product of enzymatic signal generation and activate or deactivate signal transducers such as synthetic pores.[6] Herein, we report that with the combination of counterion activation[2,3] and signal amplification methods,[6] one of the most persistent sensing problems with synthetic pores, the detection of hydrophobic analytes, can be solved with CPPs. Sensing of hydrophobic analytes is a challenge with pore sensors, because they tend to avoid the hydrophilic interior of the pore and prefer partitioning into the membrane. We demonstrate that covalent capture with hydrophilic anions produces amphiphilic anions that can activate CPP transporters. Coupled with enzymatic signal generation, this hybridization of activators and amplifiers affords cheap, fluorogenic, and interference-free CPP-based sensors for hydrophobic analytes in complex matrices.

Steroids provide attractive examples to illustrate this approach because they are notoriously insoluble and difficult

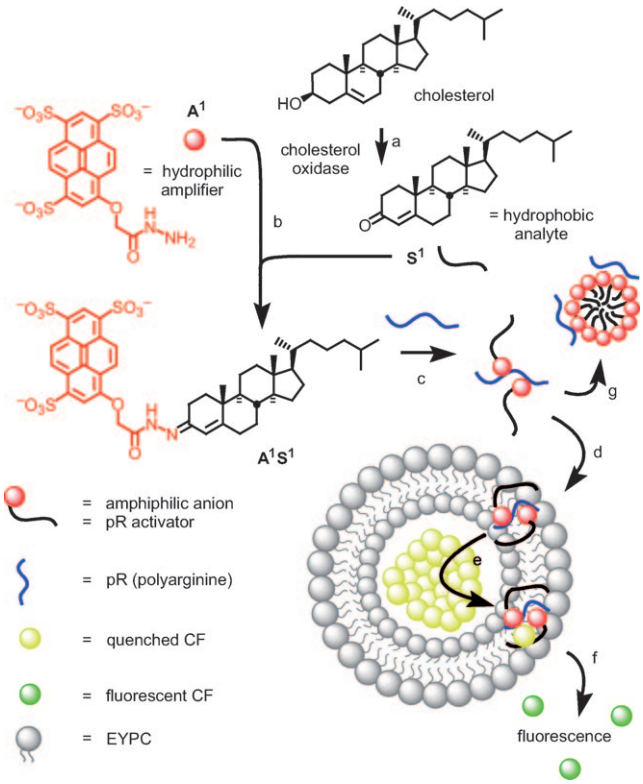

Figure 1. Fluorogenic steroid sensing systems with CPP transporters as signal transducers and signal amplifiers (e.g., $\mathbf{A^1}$) that react with hydrophobic analytes (e.g., $\mathbf{S^1}$) to generate amphiphilic CPP activators. For cholesterol, signal generation with cholesterol oxidase (a) is followed by signal amplification with $\mathbf{A^1}$ (b). The obtained $\mathbf{A^1S^1}$ conjugate and CPP (e.g., pR) form pR–$\mathbf{A^1S^1}$ transporters (c) that mediate the export of intravesicular CF (d–f). Amphiphilic anions (e.g., $\mathbf{A^1S^1}$) are CPP activators below (d–f) and CPP inactivators above their cmc (g).

[*] Dr. S. M. Butterfield, Prof. T. Miyatake, Prof. S. Matile
Department of Organic Chemistry, University of Geneva
Genève (Switzerland)
Fax: (+41) 22-379-3215
E-mail: stefan.matile@unige.ch
Homepage: http://www.unige.ch/sciences/chiorg/matile/

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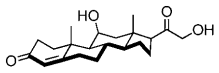
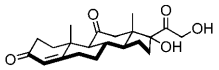
to measure in aqueous media,[7] and because their detection is essential in domains such as medicinal diagnostics[8] and environmental monitoring.[9] In the field of chemo- and biosensors,[10–12] previous approaches to address these needs included enzyme immobilization on electrochemical surfaces[11] and chemical conjugation of steroid receptors to fluorescent proteins.[12] The concept of amplifier-mediated fluorogenic steroid sensing with cell-penetrating peptides builds on the ability of pR–counteranion complexes to export CF from egg yolk phosphatidylcholine large unilamellar vesicles (EYPC LUVs, Figure 1).[2] In this assay, CF is loaded within the vesicle at concentrations high enough for self-quenching, and the local CF dilution during CF export by pR–counteranion complexes is monitored as an increase in CF fluorescence. The CF intensity is then normalized to an activity factor, the fractional activity Y.[14] To explore the compatibility of this method with reactive signal amplification strategies,[6] we used the hydrazone approach[6,13] and treated the steroid analyte $\mathbf{S^1}$ with anionic hydrazides and hydrazines $\mathbf{A^1}$–$\mathbf{A^5}$[14] in DMSO at 60 °C for one hour. Without any further sample workup or purification, the efficiency with which the resulting hydrazone amphiphiles $\mathbf{A^1S^1}$–$\mathbf{A^5S^1}$ activated pR was measured in EYPC-LUVs⊃CF. Compared to the singly charged amphiphiles $\mathbf{A^2S^1}$–$\mathbf{A^5S^1}$, the $\mathbf{A^1S^1}$ amphiphile obtained from the triply charged cascade blue (CB)[6] hydrazide $\mathbf{A^1}$ activated pR more efficiently ($EC_{50} = (14.0 \pm 1.0)$ μM), as determined from the resulting increase in fluorescence emission during the export of CF, and was clearly the best in the series (Table 1). Hydrazide $\mathbf{A^1}$ alone did not induce pR activation up to concentrations of 500 μM. For comparison, CF efflux through synthetic pores was hindered by both $\mathbf{A^1}$ alone ($IC_{50} = 22$ μM)[6] and hydrazones $\mathbf{A^1S^1}$ ($IC_{50} = 44$ μM) with similar efficiencies. Originating presumably from partial nonspecific binding of amphiphile $\mathbf{A^1S^1}$ to the hydrophobic membrane rather than the hydrophilic pore interior, this nearly negligible discrimination factor $D = 2$ was insufficient for sensing. The interference-free ($D = \infty$), sensitive, and fluorogenic detectability of steroid $\mathbf{S^1}$ thus represented the first example in which CPP-based sensors are clearly superior to synthetic pores.[2,5]

The dose response curve of $\mathbf{A^1S^1}$ activators at constant pR concentration was paraboloidal (Figure 2a, ○) rather than sigmoidal as usual. This result suggested that $\mathbf{A^1S^1}$ activators form micelles at higher concentrations; these micelles then act as hydrophilic polyanions, binding CPPs and keeping them away from the membrane (Figure 1g). The maximal activity Y_{max} in the dose response curve should thus correspond to the critical micelle concentration (cmc) of the activator (ca. 50 μM for $\mathbf{A^1S^1}$, Figure 2a, ○). This behavior is contrary to that of common surfactants such as triton X-100, which, in the absence of CPPs, is membrane-inactive as a monomer and membrane-disruptive as micelle ($EC_{50} = cmc = 200 \pm 8$ μM).

To increase the relatively modest $Y_{max} = 36\%$ of pR–$\mathbf{A^1S^1}$ complexes (Figure 2a, ○), the use of solubilizing additives was considered. In our hands, β-cyclodextrin, DMSO, dioxane, DMF, acetonitrile, and *tert*-butyl alcohol were ineffective as additives. However, submicellar concentrations of triton X-100 proved perfect to deliver activator $\mathbf{A^1S^1}$ to the membrane with minimal losses from competing precipitation from the aqueous phase. Increasing the concentration of triton X-100 up to 80 μM not only lowered the EC_{50} for $\mathbf{A^1S^1}$ to activate the subsequently added pR transporters but also raised the maximal activity of the resulting pR–$\mathbf{A^1S^1}$ complexes up to an excellent $Y_{max} = 60\%$ (Figure 2a, ●, and Table 1).

Fluorometric detection of the steroid series $\mathbf{S''}$ was possible at low micromolar concentrations (Table 1). This detection was achieved by covalent capture with $\mathbf{A^1}$ and subsequent activation of pR transporters in fluorogenic vesicles with the obtained $\mathbf{A^1S''}$ conjugate. The least respon-

Table 1: Steroid detection with pR and amplifier $\mathbf{A^1}$ in the presence (upper rows) and absence (lower rows) of nonmicellar triton X-100.[a]

	Steroid		EC_{50} [μM][b]	Y_{max} [%][c]
1		$\mathbf{S^1}$	0.6 ± 0.03	60
			14.0 ± 1.0	36
2		$\mathbf{S^2}$	18.0 ± 9.0	24
			–[d]	–[d]
3		$\mathbf{S^3}$	11.0 ± 0.4	57
			17.0 ± 2.0	12
4		$\mathbf{S^4}$	7.5 ± 0.1	39
			9.3 ± 0.2	37
5		$\mathbf{S^5}$	1.3 ± 0.07	74
			3.1 ± 0.5	65
6		$\mathbf{S^6}$	7.1 ± 0.3	74
			13.0 ± 1.0	63
7		$\mathbf{S^7}$	10.0 ± 0.2	50
			18.0 ± 0.4	30

[a] Determined from dose response curves for fluorogenic CF export from EYPC-LUVs⊃CF in the presence of pR and after hydrazone formation with one equivalent of CB amplifier $\mathbf{A^1}$ (Figures 1 and 2).[14] [b] Concentration of activator $\mathbf{A^1S''}$ needed to observe 50% of the maximal pR activity Y_{max}, data ± standard deviation. EC_{50} values depend on parameters such as vesicle concentration (decreasing with decreasing LUV concentration), lipid composition, surface potential, membrane potential, temperature, and ionic strength. [c] Maximal fluorescence emission intensity found at maximal nonmicellar activator $\mathbf{A^1S''}$ concentration relative to emission after membrane lysis with triton X-100, ± 5% error. [d] Not detectable.

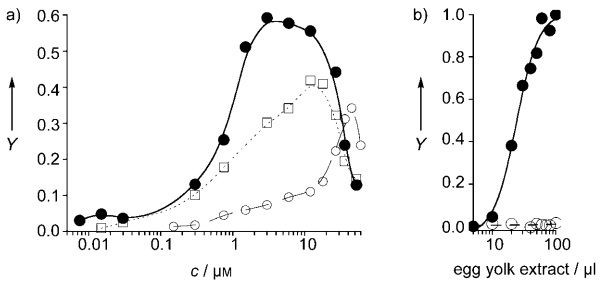


Figure 2. a) Dose response curves for pR activation with increasing concentrations c of $\mathbf{A^1S^1}$ in the presence of 0 (○), 50 (□), and 80 μM (●) triton X-100. b) Dose response curve for pR activation with increasing volumes of egg yolk extract after signal generation with cholesterol oxidase (●: 0.45 units mL^{-1}, ○: 0 units mL^{-1} (no enzyme) plus peroxidase (37 units mL^{-1}) and subsequent signal amplification with $\mathbf{A^1}$.

sive activator was estrone hydrazone $\mathbf{A^1S^2}$, for which no pR activation was observed without additives, and a Y_{max} of only 22% was reached in the presence of triton X-100 (Table 1, entry 2). The pR activating efficiencies (i.e., $1/EC_{50}$) of the steroid amphiphiles followed the order: $\mathbf{A^1S^1}$ (cholestenone) > $\mathbf{A^1S^5}$ (progesterone) > $\mathbf{A^1S^6}$ (corticosterone) ≈ $\mathbf{A^1S^4}$ (pregnenalone) > $\mathbf{A^1S^3}$ (testosterone) ≈ $\mathbf{A^1S^7}$ (cortisone; Table 1). This trend suggested that increasing hydrophilicity reduces activator efficiency. The outstanding EC_{50} values of $\mathbf{A^1S^1}$ and progesterone amphiphile $\mathbf{A^1S^5}$ were independent of number and location of the reactive ketone groups, but correlated with the maximal log *P* values of the steroids[15] and the intact hydrophobicity of their dimethylated β faces (Table 1, entries 1 and 5). The detectability of many different analytes is important to assure broad applicability of CPP sensors. The selective detection of single components in a collection of accessible analytes is achieved in this case by the selectivity of the enzymatic signal generator (cholesterol oxidase); selective detection of the other steroids in complex matrices is conceivable by appropriate choice of enzyme.

Intrinsic differences in reactivity implied that di- and triketones $\mathbf{S^5}$–$\mathbf{S^7}$ would give monohydrazone amphiphiles when treated with equimolar amounts of $\mathbf{A^1}$. ESI-MS analysis supported the in situ formation of monohydrazones $\mathbf{A^1S^5}$–$\mathbf{A^1S^7}$. The declining activation efficiency of corticosterone $\mathbf{S^6}$ with increasing molar fraction of $\mathbf{A^1}$ during incubation was consistent with the formation of the hexaanionic bolaamphiphilic species $\mathbf{A^1S^6A^1}$ with increased pR affinity but reduced affinity for the membrane. The same trend was found for triketone cortisone $\mathbf{S^7}$.

The usefulness of amplifier-mediated CPP activation to detect the activity of enzymes with hydrophobic substrates was explored next (Figure 1). Cholesterol was incubated with cholesterol oxidase, peroxidase, and triton X-100 as solubilizer for varying reaction times, treated with signal amplifier $\mathbf{A^1}$, and subsequently added with pR to EYPC-LUVs⊃CF. Increasing CF emission was found with increasing enzyme reaction time and with increasing enzyme concentration. This result was consistent with increasing concentration of pR–$\mathbf{A^1S^1}$ resulting from the enzymatic oxidation of cholesterol to $\mathbf{S^1}$.

Because of its high cholesterol content,[16] egg yolk was an ideal candidate to explore the compatibility of amplifier-mediated CPP activation with sensing applications in samples from supermarkets and hospitals (Table 2, entry 1). A dried organic extract was prepared from egg yolk following a standard protocol.[14] This extract was exposed to enzymatic signal generation with cholesterol oxidase, peroxidase, and triton X-100 solubilizer and subsequent signal amplification with $\mathbf{A^1}$. Increased volume of the resulting egg yolk extract caused increasing activation of pR transporters (Figure 2b, ●). Controls demonstrated that the egg yolk extract did not activate pR without incubation with cholesterol oxidase (Figure 2b, ○). This finding confirmed that the product $\mathbf{S^1}$ of enzymatic oxidation is the only source of pR activator in the egg yolk matrix and that subtraction of background contributions is thus redundant.[5,6] A cholesterol content of (10.2 ± 1.0) mg g^{-1} in egg yolk was obtained from comparison of the dose response curve of egg yolk extract (Figure 2b, ●) with the calibration curve for $\mathbf{A^1S^1}$ (Table 2, entry 1). This result was in excellent agreement with the typical range of 9–20 mg g^{-1} of cholesterol in egg yolk reported in the literature.

Table 2: Cholesterol sensing with pR and amplifier $\mathbf{A^1}$.[a]

Entry	Sample	Expected [mg g^{-1}][b]	Found [mg g^{-1}][c]	UV control [mg g^{-1}][d]
1	egg yolk	9–20	10.2 ± 1.0	n.d.
2	black lumpfish eggs	3.0–3.5	3.5 ± 0.5	3.5 ± 0.1
3	human blood serum	1.5–2.5	1.2 ± 0.2	1.0 ± 0.1

[a] Determined from dose response as activation of pR in fluorogenic vesicles (Figure 2). [b] From supplier/literature.[16–18] [c] Calibrated against the dose response curve of $\mathbf{A^1S^1}$. [d] Determined using a colorimetric method for cholesterol detection.[14]

Following the same procedure, cholesterol contents were determined in black lumpfish eggs[17] and human blood serum.[18] The obtained values were in agreement with expectations from the literature and independent measurements with a conventional UV assay for cholesterol (Table 2, entries 2 and 3).[14]

In summary, the present study introduces cell-penetrating peptides (CPPs) as fluorogenic multianalyte sensors in complex matrices, particularly for otherwise intractable hydrophobic analytes. The approach combines two recently described key concepts, that is, signal amplification by covalent analyte capture after enzymatic signal generation[6] and the activation of CPP transporters by anionic amphiphiles,[2,3] and it is exemplified with cholesterol sensing in blood serum and other matrices. The exceptionally broad and attractive perspectives assured by the general nature of the sensing system are currently under investigation.

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