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A Cost-Effective Method for the Optical Transduction of Chemical Reactions. Application to Hyaluronidase Inhibitor Screening with Polyarginine−Counteranion Complexes in Lipid Bilayers

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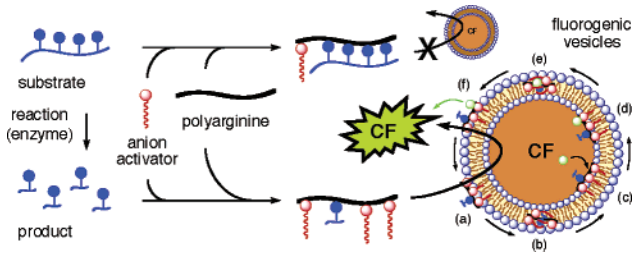
Introducing cell-penetrating peptides (CPPs)[1] as optical transducers of chemical reactions, we here report a cost-effective, simple, and quite general inhibitor-screening[2] method for enzymes, such as hyaluronidase,[3] that are of medicinal interest but otherwise difficult to address. The optical transduction of reactions with synthetic multifunctional pores has been accomplished recently.[4] We further have already demonstrated that the combination with enzymes as specific signal generators provides access to quite universal sensors.[4c] This noninvasive (i.e., label-free) and adaptable (i.e., "universal") method is broadly applicable in different fields, such as drug discovery (i.e., enzyme inhibitor screening), diagnostics (i.e., multicomponent sensing), and so on.[4d] However, the demanding synthesis of current multifunctional pores hinders commercialization and the expected widespread use. Previous attempts to replace synthetic multifunctional pores with commercially available biological pores, such as melittin, failed because of low sensitivity.[4e]

During our studies to elucidate the functional consequences of counteranion hopping on oligoarginines, we found not only that CPPs can easily cross the bilayer membrane and reach cytosol and nucleus[1f] but also that oligo- or polyarginine−counteranion complexes can act as anion carriers.[5] This finding implied that the optical transduction of chemical reactions can be achieved with commercially available material (Scheme 1). Namely, as polyarginine (pR) can transport anions, such as CF (5(6)-carboxyfluorescein), across the lipid bilayer only when it is bound to the amphiphilic "activator" anions, the presence of the competitive anions that hinder the formation of a pR activator complex should cause reduced activity. The degradation of inactivating polyanions to less inactivating mono- or oligoanions, for example, should therefore be easily detectable as an increase in the activity of pR activator complexes.

The conversion of hyaluronan (HA) by testicular hyaluronidases (HAases) to primarily tetrasaccharide products was selected as an example that may benefit from a label-free naked-eye assay.[3] HA, a high-molecular weight linear polysaccharide, is a major component of the extracellular matrix that is used in cosmetics and plastic surgery and plays, in concert with HAases, a key role in (patho)-physiological processes, such as fertilization, embryonic development, tumor growth, and metastasis. Inhibitor screening for HAases should, therefore, reveal new drugs.[2]

Counteranion screening identified dodecyl phosphate (DP) as an effective activator of polyarginine (pR) for the fluorogenic release of CF from EYPC-LUVs⊃CF ($\Delta I = I_{MAX} - I_{MIN} = 53\%$, $EC_{50}^{DP} = 19 \pm 1\ \mu M$, EYPC-LUVs⊃CF: large unilamellar vesicles composed of egg yolk phosphatidylcholine and loaded with CF).[5a] The subnanomolar HA concentrations necessary to inactivate these

Scheme 1. The Concept of Counterion-Activated Polyarginine Anion Carriers (a−f)[5] as Optical Transducers of Reactions Exemplified for the Case of Fluorogenesis by CF Release during the Consumption of Substrates, Such as the Hydrophilic Polyanion Hyaluronan



pR−DP transducers suggested unproblematic detectability of the activity of HAase (Scheme 1, Figure 1A, $IC_{50}^{HA} = 280 \pm 33$ pM).[6] For this purpose, the enzyme was incubated with the substrate HA, and aliquots were taken in meaningful intervals to monitor the ability of the reaction mixture to inhibit the release of intravesicular CF with pR−DP complexes. The found increasing pR−DP activity with increasing reaction time was as expected for consumption of the inactivator HA (Figure 1B). Compatibility of this HAase assay with inhibitor screening was readily demonstrated by decreasing velocity of HA consumption with increasing concentrations of the known HAase inhibitor, cromolyn (disodium cromoglycerate), one of the most common drugs to treat asthma (Figure 1C).[3]

With pR−DP complexes as commercially available transducers in hand, the user-friendliness of the CF vesicles was considered next. The half-life of EYPC-LUVs⊃CF prepared by the convenient

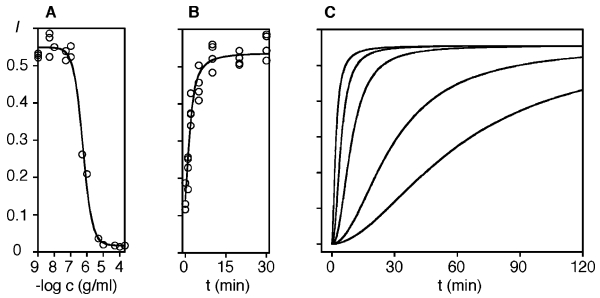


Figure 1. Application of pR−DP transducers to detect hyaluronan (A), HAase activity (B), and its inhibition by cromolyn (C) without substrate immobilization. (A) Fractional CF emission intensity I ($\lambda_{ex} = 492$ nm, $\lambda_{em} = 517$ nm) 5 min after the addition of HA (0.5 pM to 100 nM), pR (250 nM), and DP (20 μM) to EYPC-LUVs⊃CF (~25 μM EYPC, 10 mM HEPES, 107 mM KCl, pH 7.4, 25 °C); calibrated by final lysis. (B) As in (A) with reaction mixture instead of HA as a function of incubation time (13 μL in 2 mL of EYPC-LUVs⊃CF suspension; reaction mixture: 96 nM HA, 200 units/mL HAase, 100 mM NaOAc, 150 mM NaCl, 2.5 mM $CaCl_2$, pH 4.0, 37 °C). (C) As in (B) in the presence of 0, 4, 6, 8, and 10 mM cromolyn during incubation with HAase (fitted data from four experimental data points each; see Supporting Information).

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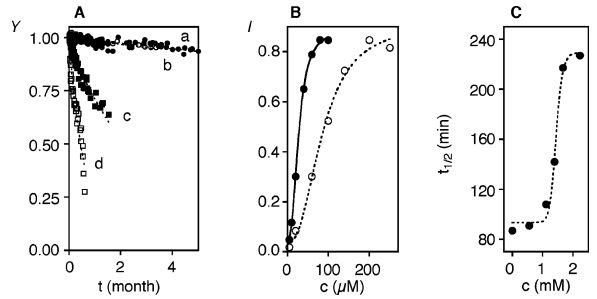
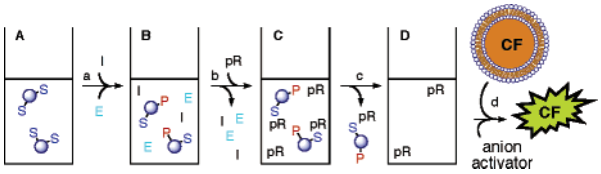


Figure 2. Application of optical pR−DP transducers in DPPC-LUVs⊃CF to characterize HAase inhibition by heparin with immobilized HA. (A) Stability of DPPC- (a, b) or EYPC-LUVs⊃CF (c, d) detected by comparing CF emission intensity [$Y = (I_{MAX} - I_{MIN})/(I_{MAX} - I_{MIN})_0$] before ($I_{MIN}$) and after lysis ($I_{MAX}$) as a function of vesicle age at 4 °C (a, c) and 25 °C (b, d). (B) CF emission I of DPPC-LUVs⊃CF (~33 μM DPPC) at 60 °C (●) and 25 °C (○) after addition of pR (250 nM) and DP (0−250 μM). (C) Half-life of HA immobilized on HMBA−PEGA (160 mg/mL) in the presence of HAase (200 units/mL) and heparin (0−2.2 mM) determined from CF emission I of DPPC-LUVs⊃CF (~33 μM DPPC, 60 °C, 80 μM DP) with residual pR obtained as outlined in Scheme 2.

Scheme 2. Application of Optical pR−DP Transducers to Inhibitor Screening with Immobilized Substrate on Solid Support[a]



[a] (A) Addition of inhibitor (I; e.g., heparin) and enzyme (E; e.g., HAase) to substrate (S; e.g., HA) on solid support (e.g., HMBA−PEGA). (B) Removal of I and E by filtration, followed by addition of pR. (C) Removal of immobilized S−pR complexes by filtration. (D) Addition of anion activator (e.g., DP) and LUVs⊃CF to measure the residual pR.

freeze−thaw−extrusion method was $t_{1/2} = 9.7$ weeks at 4 °C (Figure 2Ac). A more attractive $t_{1/2} = 3.5$ years on the shelf was estimated for DPPC-LUVs⊃CF (Figure 2Ab, DPPC: dipalmitoyl phosphatidylcholine). The drop in activity of pR−DP complexes in gel-phase DPPC-LUVs⊃CF (Figure 2B (open circles), EC_{50}^{DP} (25 °C) = 86 ± 9 μM) could be readily minimized with fluid-phase detection[5a] above phase transition (Figure 2B (solid circles), EC_{50}^{DP} (60 °C) = 28 ± 1 μM). The sensitivity toward DP → HA exchange in fluid DPPC (IC_{50}^{HA} = 600 ± 110 pM) was almost as good as that in EYPC (Figure 1A).

As far as inhibitor screening is concerned, the use of the above-described method is limited by variable interference from inhibitors. Immobilization of inhibitor or substrate seemed ideal to solve this problem.[2] To develop an interference-free screening method of solution-phase inhibitor libraries, the carboxylate-rich HA substrate was therefore immobilized on HMBA−PEGA, an alcohol-rich resin.[7,8] According to detection by pR−DP transducers, the hydrolysis of solid-phase substrate HA−HMBA−PEGA by HAase was about 50-fold slower than that in homogeneous conditions. Heparin, a polysulfonated glycosaminoglycan with anticoagulant activity, was selected as a noncompetitive HAase inhibitor[3] that is incompatible with pR−DP transducers because of its high affinity for pR.[5b] With substrate HA on solid support, however, heparin inhibition was readily detectable (Figure 2C). After incubation of inhibitor heparin and enzyme HAase with immobilized substrate HA, the resin with remaining substrate was separated from the inhibitor and the enzyme by filtration and exposed to pR (Scheme 2). After removal of the resin by filtration, the remaining amount of pR that was not bound to the solid-phase substrate could be easily quantified by addition of fluid DPPC-LUVs⊃CF with DP activators. The activity of the pR−DP transducers observed by this procedure reflects directly the concentration of consumed substrate. The found increasing half-life of the solid-phase substrate HA−HMBA−PEGA with increasing concentration of the inhibitor heparin confirmed the applicability of this procedure to inhibitor screening with substrate immobilization. This finding provided another appealing example for the practical usefulness of the method introduced here to address central topics in medicinal chemistry and beyond.[4d,6,8]

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

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(5) (a) Nishihara, M.; Perret, F.; Takeuchi, T.; Futaki, S.; Lazar, A. N.; Coleman, A. W.; Sakai, N.; Matile, S. *Org. Biomol. Chem.* **2005**, *3*, 1659−1669. (b) Although presumably more complex in reality,[1f] support for a carrier mechanism includes biphasic kinetics for oligoarginine removal with heparin from and across intact vesicles, and U-tube activity: Sakai, N.; Takeuchi, T.; Futaki, S.; Matile, S. *ChemBioChem* **2005**, *6*, 114−122. (c) Sensitivity decreases with oligoarginine length: Sakai, N.; Matile, S. *J. Am. Chem. Soc.* **2003**, *125*, 14348−14356.

(6) Significantly lower sensitivity and selectivity with pR−DP transducers compared to that of synthetic multifunctional pores were identified for more challenging systems. For example, the minimal $IC_{50}^{ATP} = 1.2$ μM and the ADP/ATP discrimination factor $IC_{50}^{ADP}/IC_{50}^{ATP} = 15$ with synthetic multifunctional pores[4c] dropped to $IC_{50}^{ATP} = 430 \pm 50$ μM and $IC_{50}^{ADP}/IC_{50}^{ATP} = 4$ with pR−DP transducers. This difference was sufficient to detect kinase activity but incompatible with binary "on/off" ATP/ADP discrimination. Studies on the limitations of the universal adaptability of optical counterion−polyion transducers are ongoing with particular emphasis on covalent in situ amplification of poorly recognized inactivating as well as activating analytes.

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(8) The detectability of substrate immobilization on solid support with pR−DP transducers identified another attractive field of application of the here reported method (see Supporting Information). Substrate immobilization is also convenient for Michaelis−Menten analysis to determine the nature of inhibition.

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