Signal amplification by conjugate addition for differential sensing with synthetic pores†

Shinya Hagihara, Hiroyuki Tanaka and Stefan Matile*

Received 7th April 2008, Accepted 13th May 2008
First published as an Advance Article on the web 20th May 2008
DOI: 10.1039/b805863f

The Michael addition of hydrazide amplifiers to short-lived orthoquinone analytes is introduced as a new method of signal amplification and proposed, together with catechol capture by boronate amplifiers, to contribute toward the discrimination of catechol-containing (*e.g.* epigallocatechin gallate) and catechol-free polyphenols (*e.g.* resveratrol) with synthetic pores.

The use of synthetic ion channels and pores[1] as multianalyte sensors[2] in complex matrices depends on the availability of reactive signal amplifiers.[3–6] Their role is to capture otherwise undetectable analytes after (or before) enzymatic signal generation and enable their recognition by responsive pores for signal transduction. Amplifier technology has been used to create pore sensors for lactate,[3] citrate,[3] glutamate,[3] and polyphenols.[4] Reactive signal amplifiers were not needed to sense sucrose,[3] lactose[3] and acetate[3] with synthetic pores, and zinc filters were already sufficient to sense phytate and IP_7.[5] Most of today's amplifiers are hydrazides.[3,4,6] They can react in mildly acidic water or polar organic solvents with analytes containing aldehydes or ketones. For polyphenol sensing, the hydrazide amplifier **1** was reacted with benzaldehyde converter **2** (Fig. 1).[4] The obtained boronate amplifier **3** was used to covalently capture catechol-containing polyphenols **4** as boronate esters **5**. With this approach, synthetic pores could sense polyphenols in green tea.[4]

Polyphenols are a broad family of natural products that occur in green tea, red wine, fruits, vegetables, chocolate, and so on.[7–14] The numerous beneficial health effects of polyphenols against aging, cardiovascular diseases, neurodegenerative diseases and cancer attract intense scientific and public attention. The realized use of boronate amplifiers for polyphenol sensing with synthetic pores is naturally limited to polyphenols that contain at least one catechol.[4] This is sufficient for polyphenol sensing in green tea, where flavan-3-ols such as (+)-catechin **4a** and their gallate esters such as the powerful antioxidant and urokinase inhibitor epigallocatechin gallate (EGCG) **4b** dominate.[7] The same is true for oligomeric antitumor proanthocyanidins **4c**[8] or flavanals such as quercetin **4d**. An important example for catechol-free polyphenols **6** is *trans*-resveratrol **6j**, a polyphenol in red wine that has been associated with the "French paradox" (*i.e.*, the possibly low incidence of coronary heart disease in southern France despite high intake of fat).[9] Resveratrol will be overlooked by synthetic pores that operate with boronate **3** as reactive signal amplifier. Here, we introduce

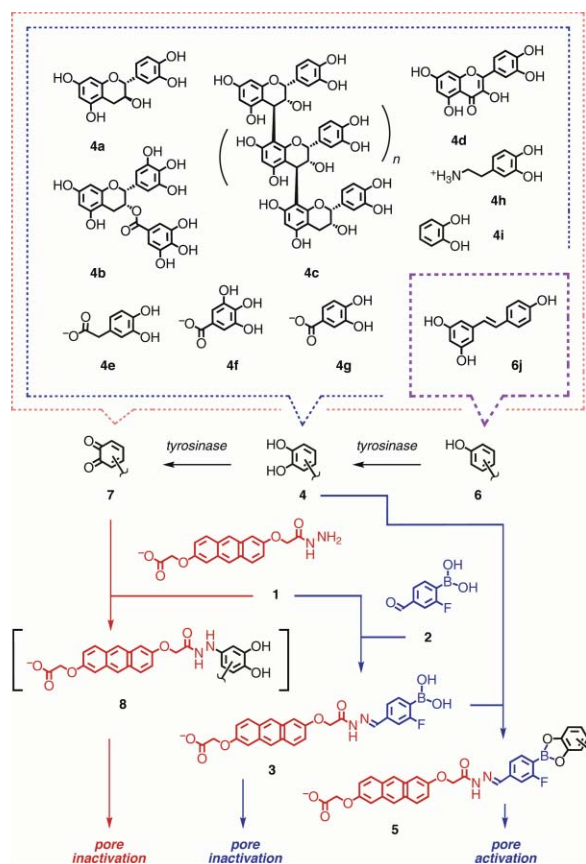

Fig. 1 The concept of differential sensing of catechol-free polyphenols **6**: Pore inactivation by product **8** of the oxidative conjugate addition of amplifier **1** to the product **7** of enzymatic signal generation with tyrosinase reveals the total polyphenol content (red), the activation of pores blocked with boronate amplifier **3** reveals the content of catechol-containing polyphenols **4** (blue), the difference should then identify the content of catechol-free polyphenols **6** such as resveratrol **6j** (purple). In general, pore inactivation is observed for increasing bulk and/or charge of amplifier conjugates (**3**, **8**). Pore activation is empirically observed for excessive bulk and/or charge of amplifier conjugates (**5**), because of size exclusion by the pore and/or preferred partitioning into the bilayer (details are often unknown). Note that product **8** and/or its oxidized orthoquinone form are just two out of a mixture of products without confirmed molecular structure.

conjugate addition of hydrazides to orthoquinones[10] as a new method for reactive signal amplification that is compatible with differential sensing of catechol-free polyphenols with synthetic pores. The short-lived orthoquinone substrates **7** are produced during signal generation with tyrosinase.[11–14] Their reaction with Michael amplifier **1** should afford pore blockers such as **8**

Department of Organic Chemistry, University of Geneva, Geneva, Switzerland. E-mail: stefan.matile@chiorg.unige.ch; Fax: +41 22 379 5123; Tel: +41 22 379 6523

† Electronic supplementary information (ESI) available: Experimental section. See DOI: 10.1039/b805863f

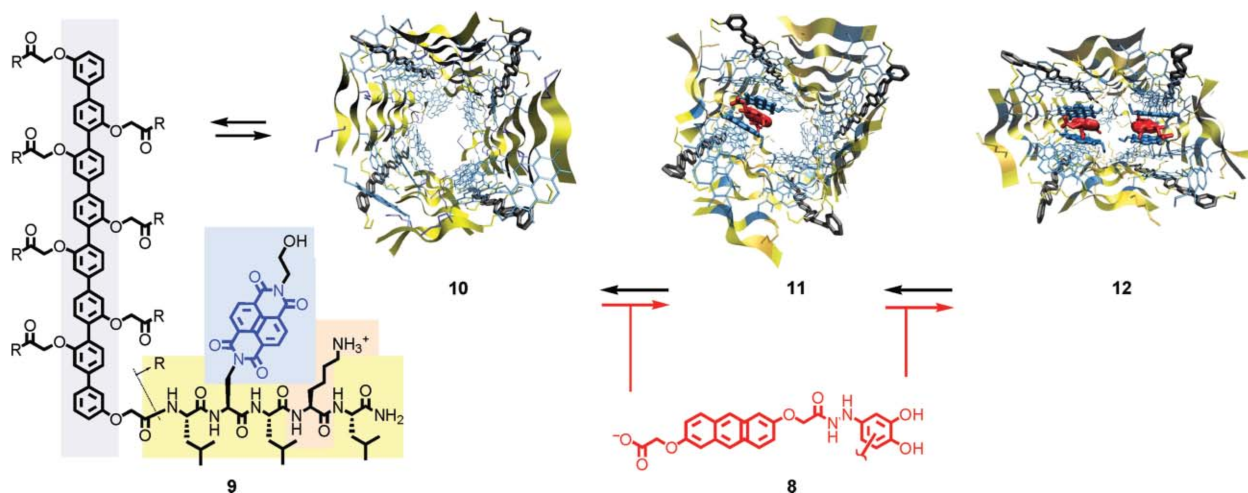

Fig. 2 Self-assembly of pore **10** from monomers **9** and molecular recognition of amplifier-analyte conjugates such as **8** (Fig. 1). The notional inclusion complexes **11** and **12** show geometry issued from molecular dynamics simulations for pore **10** with one and two blockers similar to **8**, respectively; adapted from ref. 15, © 2007, Royal Society of Chemistry.

and/or its oxidized orthoquinone form and possible degradation products. The changes in pore activity during the conversion of blocker **1** into blocker **8** should report the total polyphenol content in the sample. From this value, the value for catechol-containing polyphenols **4** obtained by reactive amplification with boronate **3** would be subtracted. The difference would then originate from catechol-free monophenolase substrates **6** such as resveratrol **6j**.

All compounds needed for an initial assessment of the validity of the concept of signal amplification by conjugate addition for differential sensing with synthetic pores were available. The synthesis of rigid-rod molecules **9**, their self-assembly into pores **10** and the use of their internal naphthalenediimide (NDI) π-clamps to recognize π-basic dialkoxyanthracene (DAA) blockers such as **8** and form inclusion complexes such as **11** with one or **12** with two blockers have been described previously (Fig. 2).[4,6,15] Synthesis and evaluation of DAA amplifier **1** have been reported as well.[6]

The sensing system was explored first with homoprotocatechuate **4e**, a flavonoid metabolite, as a simple anionic model compound for catechol-containing polyphenols **4** (Fig. 1 and 3A). The activity of synthetic pores **10** was measured in fluorogenic vesicles under standard conditions.[3–6,15] In brief, egg yolk phosphatidylcholine large unilamellar vesicles (EYPC LUVs) were loaded with 5(6)-carboxyfluorescein (CF), and activity was

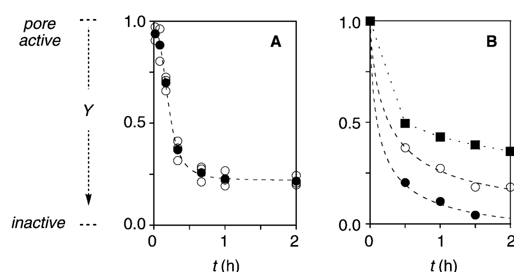

Fig. 3 Fractional pore activity Y as a function of incubation time of (A) **4e** (○, three separate measurements; ●, average) and (B) **4a** (●), EGCG **4b** (○) and resveratrol **6j** (■) with tyrosinase and **1**. The activity of pore **10** was determined in fluorogenic vesicles under routine conditions (see Fig. 4A).

monitored as increasing in CF emission with increasing CF efflux through the pore. The assay system was calibrated to high pore activity in the presence of homoprotocatechuate **4e** treated with amplifier **1** but not tyrosinase (Fig. 3A, $t = 0$). Then, homoprotocatechuate **4e** was incubated with tyrosinase in the presence of amplifier **1** to react *in situ* with the transient orthoquinone products **7e**. To the obtained reaction mixture containing the amplifier-analyte conjugates **8e**, CF vesicles and synthetic pores were added, and the ability of the reaction mixture to reduce pore activity was determined from the reduction in CF emission. The activity of pore **10** was found to decrease rapidly with increasing incubation time of homoprotocatechuate with tyrosinase and signal amplifier **1** (Fig. 3A).

(+)-Catechin **4a** as a tyrosinase substrate gave similar results, reaching full pore inactivation within two hours of incubation with tyrosinase and signal amplifier **1** (Fig. 3B, ●). Epigallocatechin gallate **4b** was confirmed as a valid but less preferred tyrosinase substrate (Fig. 3B, ○). Even catechol **4i** was identified as a surprisingly efficient pore inactivator after enzymatic signal generation with tyrosinase and *in situ* signal amplification with hydrazide **1** (not shown). However, pore **10** failed to report on gallate **4f**, protocatechuate **4g** and dopamine **4h** after incubation with tyrosinase and signal amplifier **1**. This lack of responsiveness of pore **10** to confirmed tyrosinase substrates was not surprising considering the instability of the corresponding orthoquinone products, presumably undergoing rapid polymerization[13] or intramolecular cyclization[14] before reaction with hydrazide **1**. The detectability of the neutral quercetin **4d** was not straightforward because of solubility problems.

To quantify the efficiency of individual inactivators **8**, catechol-containing polyphenols **4** were incubated for constant periods of time with tyrosinase and hydrazide **1**. Original curves for the response of pore **10** to the obtained amplifier-catechin conjugate **8a** are shown in Fig. 4A. Decreasing pore activity with increasing concentration of hydrazide **8a** was found. Hill analysis of the dose response curve gave an $IC_{50} = 77 \pm 10$ μM for (+)-catechin **8a** (Fig. 4B, ●; IC_{50} is the inhibitory concentration needed to reduce pore activity to 50%). The Hill coefficient $n = 2.6 \pm 0.7$ suggested

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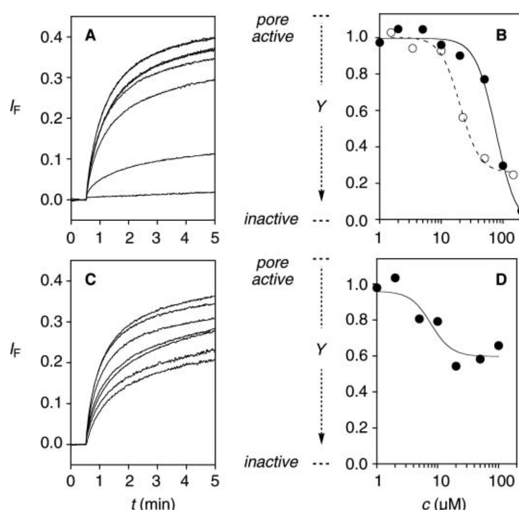

Fig. 4 Dose response curves for (B) diphenolase substrates (+)-catechin **4a** (●) and homoprotocatechuate **4e** (○) as well as for (D) monophenolase substrate resveratrol **6j**, showing the fractional activity Y of pore **10** as a function of the concentration of the analytes after incubation with tyrosinase and amplifier **1** (with fit to Hill equation). (A, C) Original data, showing fractional CF emission I_F (λ_{ex} 492 nm, λ_{em} 517 nm) of mixtures of EYPC-LUVs⊃CF (~125 μM EYPC final, 25 °C, stirred) added to (A) **4a** and (C) **4j** after incubation with tyrosinase (EC 1.14.18.1, 10 μg ml^{-1}) and amplifier **1** (50 μM, 2 h, 37 °C) during the addition of **9** (375 nM final), calibrated with final addition of excess triton X-100 (t > 5 min; all in 10 mM HEPES, 107 mM NaCl, pH 6.5).

that the inclusion of at least two blockers is needed to inactivate pore **10** (as computed in inclusion complex **12**, Fig. 2). Application of the same procedure to homoprotocatechuate **8e** gave an IC_{50} = 9.1 ± 0.9 μM (Fig. 4b, ○). Without signal amplification, the detection of homoprotocatechuate was with IC_{50} = 5.1 mM more than two orders of magnitude less sensitive. Overall, this evidence for the fluorometric detection of the diphenolase activity of tyrosinase with synthetic pores was important because it confirmed conjugate addition of hydrazides to orthoquinones as a valid new method of signal amplification for synthetic pore sensors. This is true although the short-lived product mixtures obtained from catechol oxidation are complex and the molecular structures of the obtained amplifier–analyte conjugates **8** remain to be confirmed.[11–14]

The detectability of the monophenolase activity of tyrosinase with pore **10** and amplifier **1** was explored under identical conditions with resveratrol **6j** as confirmed[12] substrate. Different to **4a**, **4b** and **4e**, pore inactivation by resveratrol **6j** after exposure to tyrosinase and amplifier **1** did not reach completion after incubation with the enzyme for two hours (Fig. 3B, ■). Many processes may account for this difference, including slower monophenolase kinetics compared to the less demanding diphenolase activity,[11] partial degradation of oxidized resveratrol before reaction with amplifier **1**,[13] a combination of both effects, poor solubility, incomplete pore blockage by the obtained product **8j**, and so on. The responsiveness of pore **10** to increasing concentrations of resveratrol **6j** treated with tyrosinase and amplifier **1** for constant periods of time was correspondingly less pronounced compared to (+)-catechin **4a** (Fig. 4, A/B *vs.* C/D). An IC_{50} = 7.8 ± 3.2 μM was found for resveratrol **8j** (Fig. 4D). This value should be appreciated with caution since not all the processes involved are fully understood and optimized. However, the reported results provide sufficient evidence to demonstrate the detectability of the monophenolase activity of tyrosinase with pore **10** as signal transducer, hydrazone **1** as signal amplifier and resveratrol **6j** as a substrate.

In conclusion, we report the fluorometric detection of monophenolase and diphenolase activity of tyrosinase with synthetic pores for various substrates. This breakthrough is important for several reasons. It introduces conjugate addition as a new and somehow biomimetic[16] method of signal amplification for synthetic pore sensors. The detectability of monophenolase activity demonstrates that this new method of signal amplification can capture otherwise undetectable, short-lived analytes. As discussed in the introduction, subtraction of the catechol-containing polyphenols measured with boronate amplifiers from the total polyphenol content measured with Michael amplifiers should, in principle, reveal the content of catechol-free polyphenols such as resveratrol (Fig. 1). Considering the additional complexity of tyrosinase chemistry,[11–14] we note that eventual sensing applications will always remain qualitative to a certain extent.[6] However, the reported results are particularly important from a more general point of view because they further advance our ability to create supramolecular functional systems of increasing complexity that do well what they are asked to do, here to execute, for the first time, a process as challenging as differential, chemoorthogonal signal amplification for synthetic pore sensors.

Acknowledgements

We thank Ludovic Gremaud and Duy-Hien Tran for contributions to pore and amplifier syntheses, Naomi Sakai and Scott A. Snyder (Columbia)[9] for advice, and the University of Geneva and the Swiss NSF for financial support.

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