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Communications

Supramolecular Tandem Assay for Pyridoxal-5′-phosphate by the Reporter Pair of Guanidinocalix[5]Arene and Fluorescein

Yu-Xin Yue,[a] Yong Kong,[b] Fan Yang,[b] Zhe Zheng,[a] Xin-Yue Hu,[a] and Dong-Sheng Guo*[a]

Dedication to Jean-Marie Lehn on the occasion of his 80th birthday

Guanidinocalix[5]arene and fluorescein reporter pair has been chosen to set up a supramolecular tandem assay principle based on the differential recognition of pyridoxal-5′-phosphate (the substrate of alkaline phosphatase, ALP), pyridoxal (the product of ALP) and phosphate (the product of ALP). This supramolecular tandem assay system offers an opportunity to monitor the activity of ALP in a label-free, continuous, and real-time manner. More importantly, a calibration curve can be given for selective and quantitative detection of pyridoxal-5′-phosphate (biomarker for several diseases).

Pyridoxal-5′-phosphate (PLP) is the bioactive form of vitamin B6.[1] Act as a ubiquitous enzyme cofactor, PLP shows an important role in many reactions in body such as racemization, transamination and elimination.[2] Plasma PLP is generally considered to be an effective diagnostic indicator of vitamin B6 status in humans.[3] Due to its function in many biological metabolic pathways, PLP can be used as a biomarker to highly metabolic cells such as cancer cells, especially to metastatic cells.[4] Therefore, PLP is demonstrated as a target molecule for tumors diagnosis, such as bladder, colon and breast cancers.[5] Besides, low level of PLP in plasma has been associated with several pathologic conditions including neonatal epileptic encephalopathy,[6] depression,[7] Alzheimer's disease,[8] rheumatoid arthritis.[9] Consequently, selectively detecting PLP is highly on demand especially with facile, low-cost and general methods.

Supramolecular tandem assay (STA)[10] is an approach for monitoring enzymatic activities, which is conceptualized by Nau and coworkers on the basis of indicator displacement assay (IDA).[11] This method depends on differential binding affinities of a macrocyclic host to the substrates and the products in a enzymatic reaction.[10a,b] The key idea of the STA is that the competitor is not added, but rather generated during an enzymatic reaction. For example, a weak competitor (substrate) can transform into a strong competitor (product) through an enzymatic reaction, and the fluorescent dye is displaced out of the macrocycle by the depletion of the substrate, *vice versa*.[10c,d] This process can be signaled by the fluorescent change with time. It makes a sensitive and real-time way to monitor the enzymatic activity.[12] Besides, enzymes are introduced to facilitate specific recognition towards analytes. As a result, STA can measure the concentrations of substrates directly as well as their fluctuations specifically.[13] The assay also offers the possibility for screening enzymatic inhibitors[14] or developing sensor arrays.[15]

In this work, we reported an approach to quantitatively detect PLP in virtue of the STA principle (Scheme 1). Guanidinocalix[5]arene (GC5 A-6C) was designed to obtain differential binding affinities to the enzymatic substrate PLP, products pyridoxal (PL) and phosphate (P). Fluorescein (Fl) was employed as the competitive dye on accounts of its strong binding with GC5A-6C, the high brightness itself and the drastic complexation-induced quenching of fluorescence.[16] Alkaline phosphatase (ALP) was engaged in degrading PLP to PL and P.[17] Therefore, this combinational system executes the ability to detect PLP.

In order to create a tandem assay, the binding affinities of the potential competitors (substrate and product) are needed to be determined by the executing IDA. The binding affinity of GC5A-6C and Fl was determined as $(7.0 \pm 2.1) \times 10^6$ M^{-1} by direct

[a] Y.-X. Yue, Z. Zheng, X.-Y. Hu, D.-S. Guo
College of Chemistry, State Key Laboratory of Elemento-Organic Chemistry, Key Laboratory of Functional Polymer Materials (Ministry of Education), Tianjin Key Laboratory of Biosensing and Molecular Recognition, Nankai University, Tianjin 300071, P. R. China
E-mail: dshguo@nankai.edu.cn
[b] Y. Kong, F. Yang
Research Institute of Petroleum Engineering, Sinopec, Beijing 100101, P. R. China
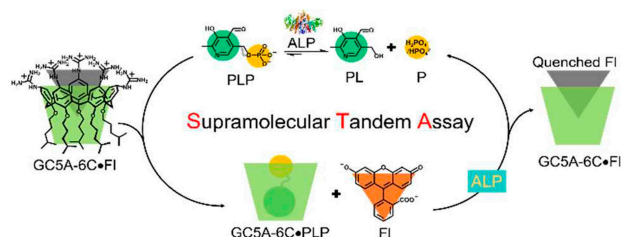
Scheme 1. Schematic illustration of STA constructed by GC5A-6C·Fl reporter pair to detect PLP.

fluorescence titration in HEPES buffer (0.01 M, pH = 7.4) containing 2 mM MgCl$_2$ (activator of ALP activity) at 37 °C, which is similar to the K_a value reported before in HEPES buffer at 25 °C.[18] Quantified by competitive fluorescence titrations, the binding affinities of enzymatic substrate PLP, products PL and P with GCA-6C were obtained (Figure 1): GC5A-6C gives a strong binding affinity to PLP with the association constant (K_a) up to $(2.0 \pm 0.5) \times 10^6$ M^{-1}, whereas a medium binding affinity to P ($K_a = (7.9 \pm 0.9) \times 10^4$ M^{-1}) and a negligible binding affinity to PL ($K_a > 00$ M^{-1}). These binding affinities indicate that the molecular recognition of GC5 A-6C with PLP is a synergistic effect: the cavity of GC5 A-6C affords the host-guest interaction towards the PL portion, and the decorated guanidinium groups afford salt bridge interactions to the P portion. Therefore, the binding affinity of corresponding ALP substrate PLP has almost 25 times

higher than product P and over 4 orders of magnitude higher than the product PL. The differential binding affinities between enzymatic substrate and products make the STA principle to be practical.

The enzymatic conversion of PLP into P and PL can be easily monitored by the fluorescent change of the GC5A-6C·Fl reporter pair (Figure 2a). Behaving as a strong competitor, PLP displaces Fl from the reporter pair, which results in a high initial fluorescence intensity. After the dephosphorization, the formed P and PL behave as weak competitors, which thereby permits the regeneration of the GC5A-6C·Fl complex with the fluorescence quenched. We selected the concentration of GC5A-6C in which it fells in the initial, approximately linear region of the GC5A-6C·Fl titration curve (Figure 1a, arrow). When working in this concentration range, the regeneration of the fluorescence intensity has a linear relationship with the concentration of titrated PLP. In a similar way, the depletion of PLP by ALP is linearly correlated to the decrease of the fluorescence intensity. Therefore, the GC5A-6C·Fl reporter pair can quantitatively convert the enzymatic reaction of ALP into a fluorescence signal which is easy to observe. The operating concentrations of PLP were selected below the Michaelis-Menten constant K_M value of ALP (Figure 2a, arrow), which allowed to investigate the enzyme kinetics. Fl is a classical probe with high brightness, and the complexation-induced quenching by GC5A-6C is drastic. These two factors enable us to monitor the enzymatic activity sensitively.

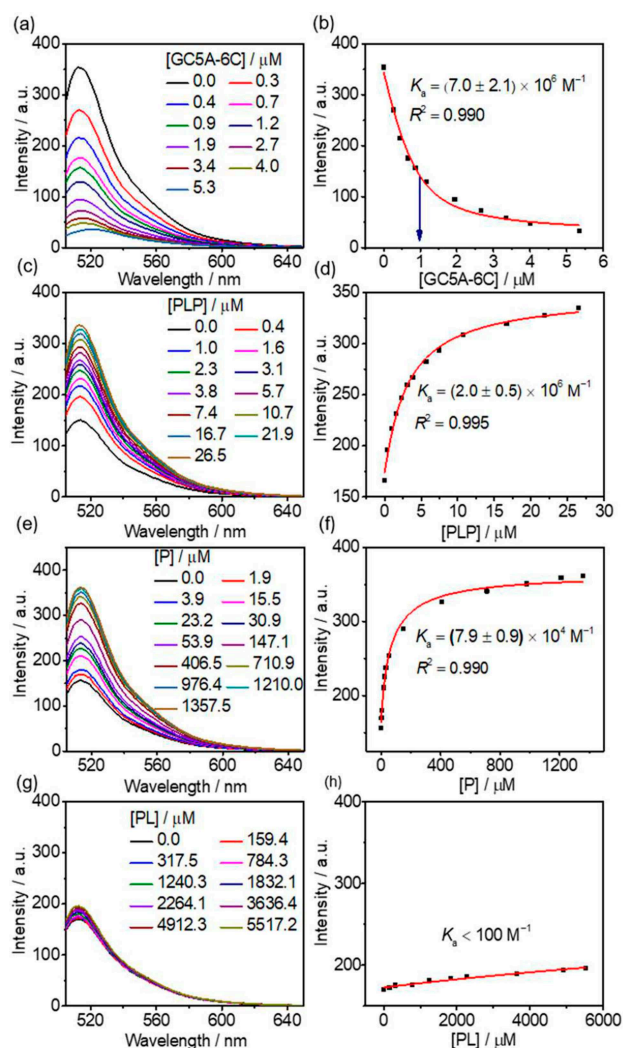As a matter of fact, the ALP assay worked exactly as planned. Figure 2b shows the corresponding kinetic

Figure 1. Direct fluorescence titration of (a) Fl (0.8 μM, $\lambda_{ex} = 500$ nm, $\lambda_{em} = 513$ nm) with GC5 A (up to 5.3 μM) and (b) the associated titration curve fitting according to a 1:1 binding stoichiometry. Competitive fluorescence titrations of GC5A-6C·Fl (0.8/1.0 μM, $\lambda_{ex} = 500$ nm, $\lambda_{em} = 513$ nm) with (c) PLP (up to 26.5 μM), (e) P (up to 1357.5 μM) and (g) PL (up to 5517.2 μM) and the associated titration curves of fluorescence titrations for (d) PLP (f) P and (h) PL fitting according to a 1:1 competitive binding model. All experiments are in HEPES buffer (0.01 M, pH = 7.4) containing 2 mM MgCl$_2$ (activator of ALP activity) at 37 °C.
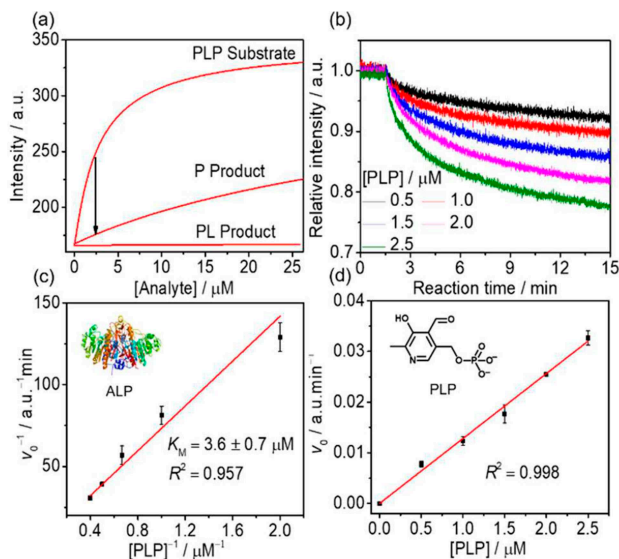
Figure 2. (a) Competitive fluorescence titration plots of PLP, PL and P with the GC5A-6C·Fl reporter pair; (b) Determination of the K_M value by monitoring ALP (1 U mL^{-1}) activity with various concentrations of PLP (0.5–2.5 μM) in the presence of GC5A-6C·Fl reporter pair (0.8/1.0 μM, $\lambda_{ex} = 500$ nm, $\lambda_{em} = 513$ nm); (c) Lineweaver-Burk plot for ALP. Error bars could not be shown if less than 1.0. (d) Linear relationship between the initial reaction rates and the concentration of PLP (0.5–2.5 μM) in the presence of ALP (1 U mL^{-1}), Fl (0.8 μM), GC5A-6C (1 μM). Error bars cannot be shown if less than 0.0005. All experiments are taken in HEPES buffer (0.01 M, pH = 7.4) containing 2 mM MgCl$_2$ (activator of ALP activity) at 37 °C.

fluorescence decay traces. The titration plots predict a maximum fluorescence decrease by ca. 28% at 0.5 to 2.5 μM substrate concentration for the ALP assay (Figure 2a), which fit well with the actual enzyme assay (decrease by nearly 25%, Figure 2b). The initial time-dependent change in fluorescence intensity is in direct ratio to the reaction rate. This allows the estimation of the K_M for ALP (Figure 2c) by varying the initial concentrations of PLP. A tentative fitting of the data gives a K_M value of (3.6 ± 0.7) μM (Figure 2c, $R^2=0.957$), which agrees well with the literature value.[19] This was achieved according to the Michaelis-Menten model (Eq. 1) by the method of initial rates, in which v_0 is the initial rate of the enzyme reaction, $[E]_0$ is the initial enzyme concentration, $[S]_0$ is the initial substrate concentration, k_{cat} is the catalytic rate constant, K_M is the Michaelis-Menten constant. Furthermore, Eq. 1 was employed to deduce the Lineweaver-Burk plot (Eq. 2). In the Lineweaver-Burk equation, $1/k_{cat}[E]_0$ is the y-intercept, and $K_M/k_{cat}[E]_0$ is the slope. When the enzymes is obeying Michaelis-Menten kinetics, the value of K_M was obtained by plotting the reciprocal of substrate concentration versus the reciprocal of velocity and then dividing the slope by the intercept.

$$v_0 = \frac{k_{cat}[E]_0[S]_0}{K_M + [S]_0} \tag{1}$$

$$\frac{1}{v_0} = \frac{K_M}{k_{cat}[E]_0} \times \frac{1}{[S]_0} + \frac{1}{k_{cat}[E]_0} \tag{2}$$

STA not only provides real-time continuous method to monitor enzymatic activity, but also offers the potential for quantitatively detecting the concentrations of analytes.[13] In a complicated condition, the simple IDA strategy may be impossible to differentiate PLP because of the existence of unknown interferences. The substrate specificity of the enzymes allows the determination of enzymatic substrate concentrations under complicated condition. Moreover, this readout is much more convenient, because the long incubation time is not essential, and the fluorescence intensity value can be readout directly after the reaction completion. The presently described integrated enzymological supramolecular method has clear-cut advantages over previous systems because of the high sensitivity and specificity. The change of the fluorescent value that results from the enzymatic reaction can be used to measure the analyte concentration. Similar as the enzyme assay above, we employed the STA strategy, in which we first added PLP to displace Fl from the reporter pair. Then ALP was added to convert PLP to PL and P, which resulted in a decrease of fluorescence intensity. As can be seen from the traces in Figure 2b, the initial conversion rate of ALP is sensitive to the concentration of PLP. When working below the K_M value of the enzyme, the working rationale behind the PLP detection is that the initial reaction rates and the concentration of substrate are directly proportional to each other. The resulting initial rates show a linear dependence on the concentrations of PLP (Figure 2d, $R^2=0.998$), which serves as the calibration curve for

selectively and quantitatively detecting PLP. The limit of detection was calculated as (26.5 ± 0.6) nM by utilizing a $3\sigma/$slope method.[20] The concentrations of PLP in human plasma are generally in the range of 10–100 nM.[21] Therefore, the present method is principally suitable for detecting PLP levels in plasma.

In summary, by utilizing the hydrolysis reaction of ALP with PLP and the binding differentiation of calixarene towards the substrate (PLP) and the products (P and PL), a successful STA principle for the selective and quantitative detection of PLP by using the GC5A-6C·Fl reporter pair was implemented. Compared with the routine analytical methods for PLP, for example, high performance liquid chromatography detection,[22] mass spectrometry,[23] and radioactive tyrosine decarboxylase assays,[24] the STA strategy is convenient, sensitive, low-cost, and offers a potential real-time monitoring way of the analytes concentrations. The obtained results have feasible implications in disease diagnosis, in which PLP is an important biomarker. Moreover, the present reporter pair shows potential application in activity assays of various protein/peptide kinases and phosphatases involved in phosphorylation and dephosphorylation, such as protein tyrosine kinase-7,[25] T4 polynucleotide kinase,[26] polo-like kinase 1,[27] protein tyrosine phosphatase,[28] and protein histidine phosphatase.[29]

Experimental Section

All the chemical materials were commercially available and used as received without further purification. Fluorescein was obtained from Tokyo Chemical Industry. Pyridoxal was purchased from 3Achem. Pyridoxal-5′-phosphate was purchased from Hua Xia Yuan Yang. Alkaline phosphatase was purchased from Takara. Dibasic sodium phosphate was obtained from Aladdin. Magnesium chloride ($MgCl_2$) solution ($1.00\ M\pm0.01\ M$) was purchased from SIGMA. GC5A-6C was synthesized according to the previous literature procedure.[18]

HEPES buffer solution preparation (0.01 M, pH 7.4): Weigh 2.38 g 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and dissolved it in ultrapure water (approximately 900 mL), then titrated to pH 7.4 with NaOH at 25 °C, the pH was determined by a pH-meter corrected using standard buffer solutions, and then made up to 1000 mL with ultrapure water. Finally, the final pH value was confirmed by the pH-meter. The HEPES buffer containing 2 mM $MgCl_2$ was prepared by the addition of the $MgCl_2$ solution (1.00 M, 80 μL) into HEPES buffer (0.01 M, 40 mL).

The fluorescence measurements were carried on a Cary Eclipse, which equipped with a Cary single-cuvette peltier accessory. It was taken in a light-path-10 mm conventional quartz cuvette. All fluorescence titrations and enzyme assays were measured in HEPES buffer (0.01 M, pH 7.4) containing 2 mM $MgCl_2$ at 37 °C. The addition of $MgCl_2$ was used as the activator of ALP activity.

The data from the direct and the competitive host-guest titrations was fitted in a nonlinear manner,[13] and the fitting pattern can be downloaded from the website of Prof. Nau's group (http://www.jacobs-university.de/ses/wnau).

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