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MOF based fluorescent assay of xanthine oxidase for rapid inhibitor screening with real-time kinetics monitoring

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ABSTRACT

The activity assay of xanthine oxidase (XO) is of great application value in clinical diagnosis because the abnormal level of this enzyme is related to a series of pathological states. In this work, a Zr based metal-organic framework (BTB-MOF) with stable photoluminescence in pure water and buffer solution was synthesized. The examination about the fluorescent responses of this material to xanthine and its oxidation product, uric acid, showed that, although both of them affected the emission of BTB-MOF in quenching form, the efficiencies presented much difference. Taking advantage of this feature, a fluorescent method was developed for the activity assay of XO, that is, BTB-MOF was added to the enzymatic oxidation system as a sensor to transduce the proceeding of the reaction real-timely to the signal of fluorescent intensity change. Our method can work under the interference of normal biologically related species, and precisely reflect XO activity in the range of 0.2–40 U L^{-1} (detection limit = 0.004 U L^{-1}). With consecutive fluorescence intensity scan, this assay could be applied as a high speed screening method of XO inhibitors with the testing time of 1 min. This work shows the wide potential application of MOFs in enzyme analysis.

1. Introduction

Xanthine oxidase (XO) is a form of xanthine oxidoreductase that catalyzes the oxidation of hypoxanthine to xanthine and subsequently to uric acid using O_2 as oxidant [1]. This enzyme shows broad substrate specificity and also participates in the catabolism of other purines [2]. An elevation in the level of XO results in a condition known as gout, which is a common rheumatic disease and an acute inflammatory arthritis [3]. Generating superoxide and H_2O_2 in the oxidation process, XO is also a major endothelial source of oxidative stress in the vasculature [4]. Accumulating evidences have been proposed to suggest the association of XO with vascular diseases, liver damage and chronic heart failure [5]. Due to these points, the activity assay of XO and the evaluation of the inhibitory effects of compounds on XO are of great significance in clinical diagnosis and drug development.

Various methods have been developed for the activity assay of XO. The better known and often used assays for this enzyme include manometric [6], colorimetric [7], fluorometric [8], and chromatographic [9] methods. Among them, the fluorometric assay is especially suitable for the analysis of small concentrations in crude tissue preparations due to the advantage in sensitivity [8]. However, the present fluorometric method uses 2-amino-4-hydroxypteridine as substrate, which is not the primary substrate for XO and shows obviously lower affinity with enzyme than that of xanthine [10], so the results obtained by this method are less representative. Therefore, developing a fluorometric assay that can authentically reflect the activity of XO towards natural substrates is still in urgent need.

With incomparable composition flexibility and extended porous structure, metal-organic frameworks (MOFs) are emerging in recent years as a kind of promising fluorescent sensing materials [11,12]. The application of MOF to enzyme analysis has also been tentatively explored [13,14]. In our previous work, a new MOF based sensing mode was proposed for the activity assay of enzyme [15]. This assay strategy needs a MOF that only exhibits fluorescent response to the substrate or the product of enzymatic reaction. Adding this MOF to the reaction media could transduce the proceeding of the reaction real-timely to fluorescent signal. In this research, we extend this strategy, and show that, even both of the substrate and product influence the emission of MOF in the same direction, a medium difference in the susceptibility to them could guarantee the application of MOF to follow the enzymatic reaction. An aromatic MOF (BTB-MOF), whose fluorescence is sensitive to uric acid but relatively inert to xanthine, was successfully applied to

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the activity assay of XO.

2. Experimental

2.1. Materials and general methods

All chemicals were obtained commercially and used as received without further purification. The aqueous suspensions used in fluorescence measurements were prepared with ultrapure water.

Powder X-ray diffraction (PXRD) pattern was recorded by a Rigaku D/max-2500 diffractometer with Cu Kα radiation (λ = 0.15406 nm) at 40 kV and 100 mA. Elemental analysis (C, H and N) was performed on a Perkin-Elmer 240C analyzer. Thermogravimetric analysis (TGA) was operated with a Rigaku standard TG-DTA analyzer from ambient temperature to 700 °C with a heating rate of 10 °C min^{-1} in the air, and an empty Al_2O_3 crucible was used as the reference. Fourier transform infrared (FT-IR) spectroscopy was measured by a Nicolet Avatar 370 Fourier transform infrared spectrometer with nujol mull method. Scanning electron microscopy (SEM) images were taken by a JEOL JSM-7500F scanning electron microscope. Steady state fluorescence experiments were carried out on an Agilent G9800A fluorescence spectrometer equipped with a SPVF-1 × 0 accessory to control the temperature. Absorption spectra were recorded by a Shimadzu UV-2450 spectrophotometer. Fluorescence lifetimes were measured by an Edinburgh FLS920 spectrometer employing the time correlated single photon counting technique with the time resolution of 0.5 ns, and 1000 counts were collected for each measurement.

2.2. Synthesis of BTB-MOF

BTB-MOF was synthesized generally according to the literature procedure [16]. In brief, 0.1 g $ZrCl_4$, 0.1 g 1,3,5-tris(4-carboxyphenyl) benzene (H_3BTB) and 10 mL concentrated hydrochloric acid were added sequently to 10 mL of DMF. This mixture was sonicated for 10 min, and then heated at 120 °C in a 100 mL Teflon-lined stainless steel autoclave for 48 h. After cooling down to room temperature, the formed precipitate was separated by filtration, washed with DMF and water, and dried at 60 °C for 6 h. Anal. Calcd. (%) for $[Zr_6(\mu_3\text{-}O)_4(\mu_3\text{-}OH)_4(BTB)_2(OH)_6(H_2O)_6]\cdot 3(Me_2NH_2)\cdot BTB$ (Mw = 2333.9): C, 44.82%; H, 4.01%; N, 1.76%. Found: C, 44.67%; H, 3.76%; N, 1.74%.

2.3. Fluorescent assay

The powder of BTB-MOF (5 mg) was immersed in 500 mL Tris-HCl buffer (20 mM, pH = 8.0) and dispersed by ultrasonic apparatus for 1 h. Then let it stand for a day to get a stable suspension (0.01 g L^{-1}). 3 mL of the suspension was used in each fluorescent measurement and placed in a 1 × 1 cm^2 optical quartz cuvette with continuous stirring at 37 °C in air atmosphere. After the sequential addition of xanthine and XO, the fluorescent intensity at 372 nm was continuously monitored.

3. Results and discussion

3.1. Characterization of BTB-MOF

BTB-MOF was prepared from the solvothermal reaction H_3BTB and $ZrCl_4$. The framework structure of the obtained sample and its phase purity were confirmed by PXRD analysis, in which all the diffraction peaks appeared at nearly identical positions with those of the simulated pattern (Fig. 1a). These peaks are well-defined, showing the good crystallinity of this sample. By combining the results of elemental and thermogravimetric analyses (Fig. S1), we determined the formula of BTB-MOF to be $[Zr_6(\mu_3\text{-}O)_4(\mu_3\text{-}OH)_4(BTB)_2(OH)_6(H_2O)_6]\cdot 3(Me_2NH_2)\cdot BTB$ (Mw = 2333.9). FT-IR spectrum also corroborated the presence of $Me_2NH_2^+$ ion with the absorption peaks at 2860 (ν (CH_3)), 2933 (ν (CH_3)) and 3068 (ν (NH)) cm^{-1} (Fig. 1b) [17]. Carboxyl groups gave intensive peaks at 1413 and 1595 cm^{-1}, but not in the range of 1690–1730 cm^{-1}, indicating that H_3BTB are completely deprotonated upon the formation of MOF structure. SEM observation showed that BTB-MOF presents a flower-like morphology which is piled up by irregular sheets with thicknesses of 30–45 nm and widths of 0.27–0.53 μm (Fig. 1c and d). This hierarchical structure extends the external surface of MOF particle and thus facilitates its contact with the analyte in the following sensing experiments.

3.2. Photoluminescence property and responses

The photoluminescence of BTB-MOF was examined directly with its aqueous suspension (Fig. 2a). Upon the excitation at 295 nm, BTB-MOF gave a prominent fluorescence band with the maximum at 372 nm. Its close position with that of free H_3BTB molecule (360 nm) indicates that this emission band originates from ligand-centered electronic transition. As other Zr(IV) and carboxylate ligand constructed MOFs [18,19], BTB-MOF has good stability in aqueous medium due to the strong Zr–O bonds. Immersion of the as-prepared sample in pure water or Tris-HCl buffer (pH = 8.0) for 12 h caused no remarkable structural or spectral change (Fig. S2).

The excellent photoluminescent property and high water stability of BTB-MOF got us to apply it to the activity assay of XO. Since xanthine and uric acid are the associated biomolecules of XO, firstly, we tested the fluorescent responses of BTB-MOF to these two compounds. Although both of them influenced the fluorescence of BTB-MOF in quenching form, the efficiencies showed much difference (Fig. 2b and S3). Upon the addition of uric acid to MOF suspension, the fluorescent intensity dropped sharply with the increasing of addition amount. Only 100 μM of uric acid could cause a quenching percentage of 85%. In contrast, the quenching ability of xanthine is only moderate. The addition of the same amount of xanthine could only weaken the emission of BTB-MOF by half.

To explain the difference in the quenching efficiency between these two compounds, we examined their absorption spectra. As shown in Fig. S4, uric acid gave generally higher UV absorption than that of xanthine, especially at the excitation wavelength of 295 nm in fluorescent experiments ($\varepsilon_{\text{uric acid}}$ = 11270 M^{-1} cm^{-1}, $\varepsilon_{\text{xanthine}}$ = 1770 M^{-1} cm^{-1}). This result implies that the higher quenching efficiency of uric acid on BTB-MOF could be attributed to an inner filter effect (IFE), which is caused only by the overlapping between the absorption spectrum of the analyte and the excitation and/or emission bands of the fluorophore [20]. When uric acid was added to the suspension, it would compete the excited energy against BTB-MOF and thus cause the decrease of fluorescence intensity. In this mechanism, the quenching efficiency is directly determined by the absorption intensity of the analyte at the excited wavelength of sensor, so the much higher extinction coefficient of uric acid at 295 nm than that of xanthine makes BTB-MOF give different response sensitivities to these compounds. To confirm the IFE mechanism, transient fluorescent experiments were carried out (Fig. S5). It was observed that the decay lifetime of BTB-MOF was hardly changed by the addition of different amounts of xanthine or uric acid (τ_0 = 13.45 ns, τ = 13.35–13.63 ns and 13.34–13.47 ns for the addition of 50–200 μM xanthine and uric acid, respectively), which is consistent with the feature of IFE. The IFE mechanism is a useful strategy for the design of novel sensors by converting the analytical absorption signals to fluorescence signals [21]. It has been proven that this mechanism can give the sensitivity and selectivity comparable to other quenching mechanisms [22]. More importantly, IFE does not need the contact between sensor and analyte, and thus give high response speed. Therefore, the fluorescent sensor developed with IFE mechanism is extremely suitable to kinetic analysis.