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A New Fluorometric Turn-On Detection of L-Lactic Acid Based on the Cascade Enzymatic and Chemical Reactions and the Abnormal Fluorescent Behavior of Silole

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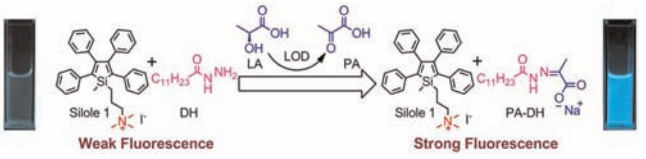
ABSTRACT



By making use of the aggregation-induced emission feature of silole 1 and the cascade reactions among L-lactic acid (LA), lactate oxidase (LOD), and dodecanoic hydrazine (DH), a new fluorometric "turn-on" method is developed for the detection of LA.

L-Lactic acid is one of the most important metabolites in clinical analysis and the food industry. Its detection is an important clinical assay for the diagnosis of numerous human disease conditions. For example, the level of L-lactic acid (LA) increases under hypoxia, poor perfusion of tissue, acute circulatory shock, and liver failure; what is more, a severe (10-fold) increase is a prime indicator of lethal risk.[1] The detection of L-lactic acid serves as an aid for diagnosing heart disease, exercise physiology, and neonatology studies.[2] Lately, it was reported that L-lactic acid can enhance the motility of tumor cells and inhibit migration and cytokine release.[3] In the food industry, the level of L-lactic acid is regarded as an indicator of the mentative process and is relevant to freshness, stability, and storage quality of food products such as wine, cider, beer, and milk.[4]

Various detection methods for LA have been reported.[5] The widely used methods are based on the enzymatic reactions. For instance, lactate dehydrogenase (LDH) with the cofactor NAD^+ was employed to transform LA to pyruvic acid (PA) with the formation of NADH which can be detected by either absorption[6] or fluorescence.[7] Alternatively, the oxidation of LA into PA catalyzed by lactate oxidase (LOD) leads to formation of hydrogen peroxide which can oxidize a chromogen in the presence of horseradish peroxidase and forms a dye complex which can be

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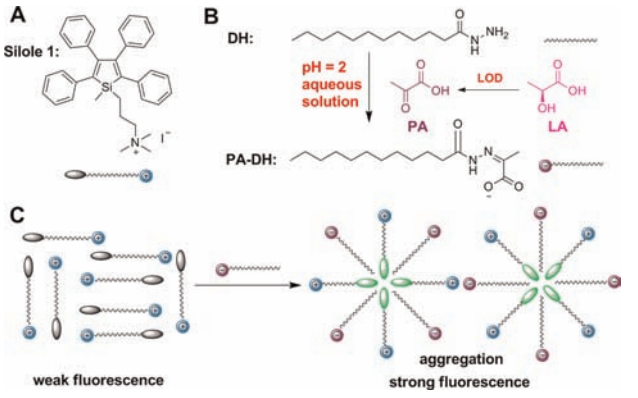
detected spectroscopically.[8] In recent years, amperometric biosensors for LA have gained more attention.[9] These biosensors are generally based on the transformation of LA in the presence of LOD and oxygen into PA and hydrogen peroxide which can be amperometrically analyzed. They are usually performed with LOD immobilized electrodes. Other electroactive compounds such as glucose, ascorbate, urate, and cysteine can interfere with the detection of LA with these biosensors, and different approaches have been invented to eliminate the interferences of these electroactive compounds.[9a,g,10] Detection of LA was also described by combining gas chromatography (GC),[11] capillary electrophoresis (CE),[12] or high-performance-liquid chromatography (HPLC)[13] with appropriate chemical derivation. In comparison, direct fluorometric detection methods for LA are rather limited. Therefore, the development of more convenient, selective, and sensitive fluorescent assays for LA is highly desired. In this paper, we will report a fluorescence turn-on assay for LA in aqueous solution by making use of the cascade enzymatic and chemical reactions and aggregation-induced emission (AIE) feature of silole (silacyclopentadiene) compounds.

Scheme 1. (A) Chemical Structure of Silole **1** with an Ammonium Group; (B) Chemical Structure of DH and LOD Mediated Reaction from LA to PA; (C) Illustration of the Design Rationale for the Fluorescence Turn-on Detection of LA by Taking Advantage of the AIE Feature of Silole Compounds



The design rationale for this new fluorometric detection of LA is schematically illustrated in Scheme 1 and is explained as follows: (1) Silole **1** with a positive ammonium group shows weak fluorescence in aqueous solution as reported earlier.[14] But, in the presence of an amphiphilic compound with a negatively charged group, aggregation would occur due to the intermolecular electrostatic and hydrophobic interactions; as a result the fluorescence of silole **1** is switched on. In fact, by taking advantage of such abnormal fluorescent behavior of silole, various chemo-/biosensors were described by us and others.[14,15] For instance, we have successfully established a fluorescence turn-on detection of heparin in serum,[14] label-free fluorometric assay for nuclease[16] and MAO-B[17] as well as their inhibitor screening, and fluorescent detection of cyanide in aqueous solution.[18] (2) Oxidation of LA catalyzed by LOD yields PA, which can react with dodecanoic hydrazine (DH) to form the respective Schiff base, an amphiphilic compound with a carboxylic acid group. It is expected that such an amphiphilic compound will form coaggregates with silole **1** via electrostatic and hydrophobic interactions in aqueous solution; accordingly, the fluorescence of silole **1** is turned on. The results reveal that a fluorescence turn-on detection of LA in aqueous solution can be established with silole **1** and the cascade reactions among LA, LOD, and DH.

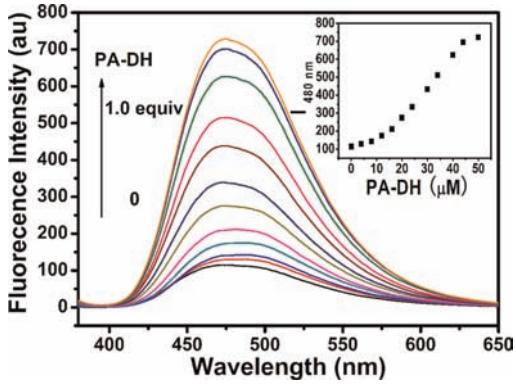


Figure 1. Fluorescence spectra of the ensemble of silole **1** [50 μM in a mixture of water and DMSO (98/2, v/v)] in the presence of different concentrations of PA-DH (from 0 to 1.0 equiv); inset shows the plot of fluorescence intensity of compound **1** at 480 nm vs the concentration of PA-DH.

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First, we demonstrate that the Schiff base from PA and DH can induce the aggregation of silole **1** leading to fluorescent enhancement. The Schiff base (PA-DH) was obtained by refluxing PA and DH in ethanol (see the Supporting Information). Silole **1** was prepared according to the reported procedures.[14] Figure 1 shows the fluorescence spectra of silole **1** after addition of different amounts of the Schiff base (PA-DH). As anticipated, silole **1** was weakly emissive in H_2O/DMSO (98:2, v/v) solution. However, upon the addition of the amphiphilic Schiff base, the fluorescence intensity of the solution increased gradually. For instance, the fluorescence intensity at 480 nm of silole **1** (50 μM) was enhanced by 7.0 times when the concentration of the Schiff base in the ensemble reached 50 μM. Interestingly, the fluorescence intensity of silole **1** increased almost linearly when the concentration of the Schiff base (PA-DH) was in the range of 0−16 μM as displayed in the inset of Figure 1.

According to previous studies,[14−18] such fluorescence enhancement observed for silole **1** should be ascribed to the aggregation of silole **1** in the presence of the amphiphilic Schiff base. The dynamic light scattering (DLS) result revealed the formation of particles of size around 1000 nm (see Figure S1) for the ensemble of silole **1** (50 μM) and 1.0 equiv of the amphiphilic Schiff base. The formation of such coaggregates of silole **1** and the Schiff base is probably due to the electrostatic and hydrophobic interactions based on previous reports.[16−18]

In the following, we want to demonstrate that the ensemble of silole **1** and DH can be employed for the detection of LA. As shown in Scheme 1, oxidation of LA catalyzed by LOD yields PA which can react with DH to form the amphiphilic Schiff base. The formation of this Schiff base was confirmed by mass spectroscopic (see Figure S6), HPLC (see Figure S7), and TLC (see Figure S8) analysis.

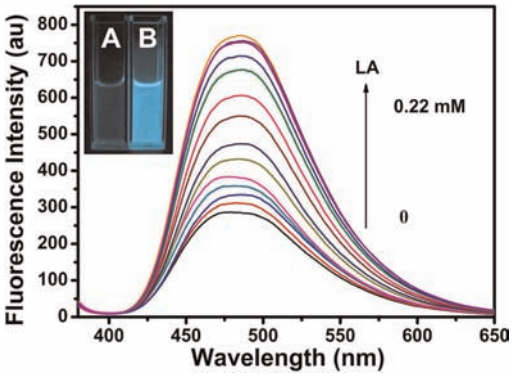

Figure 2. Fluorescence spectra of the ensemble of silole **1** (50 μM) and DH (0.3 mM) containing LOD (0.25 U/mL) in the presence of different amounts of LA (0−0.22 mM).[20] The inset shows the photos of the corresponding ensemble solution in the absence (A) and presence (B) of LA (0.22 mM) under UV light (365 nm) illumination.

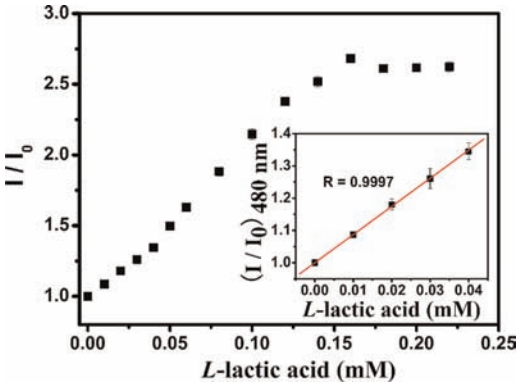

Figure 3. Plot of fluorescence intensity ratio (I/I_0 at 480 nm) vs the concentration of LA; the inset shows the plot in the concentration range of 0−40 μM for LA; I_0 represents the fluoresence intensity of the solution containing silole **1** (50 μM), DH (0.3 mM), and LOD (0.25 U/mL).

As mentioned above, the aggregation of silole **1** can occur in the presence of the amphiphilic Schiff base and as a result the fluorescence of silole **1** is switched on. Figure 2 shows the fluorescence spectra of the ensemble of silole **1** (50 μM) and DH (0.3 mM) containing LOD in the presence of different amounts of LA.[20] For comparison, the fluorescence spectra of silole **1** in the presence of DH (0.3 mM), LOD (0.25 U/mL), or PA (0.22 mM) were also measured (see Figure S4). The results manifested that the fluorescence of silole **1** was only slightly enhanced after the addition of DH (0.3 mM), LOD (0.25 U/mL), or PA (0.22 mM). However, the fluorescence intensity of silole **1** started to increase after further addition of LA as depicted in Figure 2; moreover, the fluorescence intensity increased gradually by increasing the amounts of LA (see Figure 2). In fact, such fluorescence enhancement can be distinguished by the naked eye as illustrated in the inset of Figure 2 where photos of solutions without and with the addition of LA under UV (365 nm) light illumination were displayed. Figure 3 depicts the plot of the relative fluorescence intensity (I/I_0) of silole **1** at 480 nm vs concentration of LA. Interestingly, the fluorescence intensity of silole **1** increased almost linearly with the concentration of LA in the range of 0−40 μM as displayed in the inset of Figure 3.

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(19) The activity of lactate oxidase is high in solutions of pH = 6.5−7.4; see ref 5h.

(20) After examing the influences of pH, reaction time, and temperature as well as the concentration of LOD (see Figures S2 and S3) on the enzymatic and chemical reactions, the following procedures were adopted to measure the fluorescence spectra of silole **1**: (1) different amounts of LA and LOD (0.25 U/mL) in deionized water (1.0 mL, pH $\cong$ 6.5)[19] were incubated at 30 °C for 5.0 min; (2) 5.0 μL of HCl (2.0 M) and 10.0 μL of DH (0.03 M in DMSO) were added to the above aqueous solutions which were further incubated at 30 °C for another 15 min; (3) each of the above solutions was thoroughly mixed with 10.0 μL of silole **1** (5.0 mM) in DMSO and 2.0 μL of NaOH (6.0 M) before recording the fluorescent spectrum of each solution.

Accordingly, the detection limit of LA was estimated to be 9.2 μM ($n = 11$ and $S/N = 3$).

The observed fluorescence enhancement is attributed to the cascade enzymatic (lactate oxidase-catalyzed oxidation of LA into PA) and chemical (the reaction between PA and DH) reactions to generate the amphiphilic Schiff base which can induce the aggregation of silole **1**. The fluorescence confocal laser scanning images (CLSM) support this conclusion. Figure 4 shows CLSM images of the solution containing silole **1**, DH, and LOD in the absence and presence of LA. Clearly, fluorescent aggregates were formed after the addition of LA, and almost no fluorescent aggregates were observed in the absence of LA.

Figure 4. Fluorescence confocal laser scanning images of the ensemble of silole **1** (50 μM), DH (0.3 mM) and LOD (0.25 U/mL) in the absence (a) and presence (b) of LA (0.22 mM).[20]

The interferences of saccharides, amino acids, and ascorbic acid on the detection of LA were examined with this fluorometric assay. Each of these compounds was treated with LOD and mixed with silole **1** and DH in the same manner as that for LA, followed by recording the fluorescence spectrum for each solution. The results revealed that the fluorescence of silole **1** was still rather weak after treatment with these compounds (see Figure S5). Thus, interferences from these compounds can be eliminated. This is due to the selectivity of LOD toward LA. For the application of this fluorometric assay for the detection of LA in real biological samples, it is expected that the fluorescence of silole **1** will be enhanced after mixing with samples which contain biomolecules with negatively charged moieties. But, in such cases the fluorescence intensity of silole **1** and samples without the addition of LOD and DH is first measured; then, the same amout of samples are treated sequentially with LOD and DH as described above, followed by mixing with the amount of silole **1** and recording the fluorescence intensity. The fluorescence intensity difference between these measurements can be related to the amount of LA in the sample.

In summary, we have successfully established a method of fluorescence turn-on detection of LA by making use of the abnormal fluorescent behavior of silole compounds and the cascade enzymatic and chemical reactions among LA, LOD, and DH. This fluorometric turn-on detection of LA has the following features: (1) silole **1** is easily synthesized, and DH is commercially available; (2) although the detection involves cascade reactions, the measurement can be carried out under mild conditions; (3) LA with a concentration as low as 9.2 μM can be detected, and interferences from saccharides, amino acids, and ascorbic acid are negligible.

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Supporting Information Available. Synthesis and characterization; DLS data; fluorescence spectra and relevant data for the ensemble. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.