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Selective Fluorescent Detection of Flavin Adenine Dinucleotide in Human Eosinophils by Using Bis(Zn^{2+}-Dipicolylamine) Complex

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Flavin adenine dinucleotide (FAD) is an important coenzyme in many biological systems,[1] and it is involved in numerous redox processes of metabolic reactions and biological electron transport. For example, FAD is an essential component of the electron transport chain (ETC) of the mitochondria and chlorophylls. It has been reported that the human white blood cell—eosinophil—has a considerable amount of FAD in its granules,[2] and the FAD in eosinophils plays an important role in redox reactions to generate hydrogen peroxide or superoxide that can damage a parasitic organism.[2a] The essential part of FAD in these biological reactions is an isoalloxazine ring. This isoalloxazine ring exhibits a strong fluorescence emission ($Q = 0.26$) in the green spectral region (excitation, 445 nm; emission, 525 nm).[3] The ring is also a part of flavin mononucleotide (FMN)[1d] and riboflavin (vitamin B_2),[1e] which are widely involved in many redox reactions within the cell (Figure 1). Therefore, the flavins (FAD, FMN, and riboflavin) containing the isoalloxazine nucleus exhibit similar fluorescence emission spectra in water owing to the presence of the isoalloxazine unit.

Capillary electrophoresis (CE) techniques have been developed to sensitively detect these flavins.[4] However, CE cannot be applicable to the real-time or in vivo detection of flavins. Hence, it is necessary to develop a highly selective and sensitive molecular probe for flavins under physiological conditions.

There have been many studies that used synthetic molecules for the molecular recognition of the isoalloxazine ring. Yano[5] and Rotello[6] developed synthetic receptors that can recognize the isoalloxazine ring of flavins based on hydrogen bonding, $\pi-\pi$ stacking, and electrostatic forces in organic solvents. König employed a method to detect the isoalloxazine ring using Zn^{2+}-cyclen-phenothiazine conjugate in water.[7] RNA-aptamers were developed as FAD receptors.[8] However, these compounds showed no or negligible selectivity for riboflavin, FMN, and FAD. Furthermore, to the best of our knowledge, no study has reported the development of a small molecular probe that is sensitive and highly selective, especially for FAD. Here, we report a small molecular probe that enhances only FAD fluorescence by using the conformational change of FAD before and after complexation with a dinuclear Zn^{2+} complex (**1**) of 1,3-bis[(bis(2-pyridylmethyl)-amino)methyl] benzene (see Chart 1).

The intrinsic fluorescence of FAD in an aqueous solution is considerably lower ($Q = 0.03$)[3] than that of FMN and riboflavin ($Q = 0.26$)[3] because the adenine moiety of FAD is intramolecularly stacked on the isoalloxazine ring in water, and the fluorescence of the isoalloxazine ring is almost completely quenched.[9]

Bis(Zn^{2+}-DPA) (DPA = dipicolylamine) complexes are known to be strongly bound to phosphate or a phosphodiester group in water.[10] **1** was easily synthesized in two steps with 95% yield (see the Supporting Information).

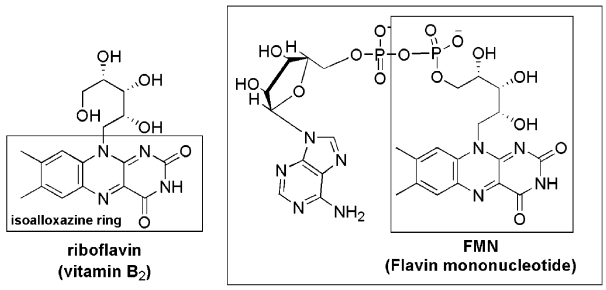
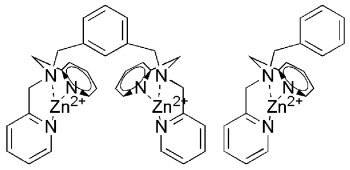


Figure 1. FAD, FMN, and riboflavin.

Chart 1. Molecular Probes for FAD



All the photophysical experiments with flavins were performed in water (10 mM HEPES, pH 7.4). Changes of FAD fluorescence emission with **1** are shown in Figure 2A. Fluorescence of FAD (50 μM) (within a few seconds) increased immediately upon addition of **1.** The fluorescence intensity at 525 nm was increased approximately 6-fold upon addition of 2 equiv of **1** (Figure 2A), and the quantum yield of FAD was enhanced by approximately 7-fold from 0.03 to 0.22, which is similar to that of FMN or riboflavin in water. The enhanced FAD fluorescence could be easily detected by the naked eye under a UV lamp at 365 nm (Figure 2B).

However, riboflavin (50 μM) shows almost no changes in fluorescence emission upon addition of 8 equiv of **1**. Fluorescence of FMN (50 μM) that possesses a phosphate group on C5 of the ribityl chain was slightly quenched upon the addition of 8 equiv of **1** (Figure 4).

Fluorescence of FAD showed a slight increase upon addition of **2**, which has only one Zn^{2+}-DPA complex. The association constant of **1** was evaluated to be 1.11×10^5 M^{-1}, but that of **2** was extremely low and could not be evaluated in water. These results indicate that the double Zn^{2+}-DPA complexes of **1** were much more tightly bound to FAD than one Zn^{2+}-DPA complex of **2**. Job's plot for the binding between **1** and FAD shows a 1:1 binding stoichiometry (see the Supporting Information). ^{1}H NMR and UV data indicate that binding of **1** to the diphosphate moiety of FAD forces the adenine and isoalloxazine rings apart (see the SI).

1 was used as a probe that targeted the FAD present in large amounts in the eosinophil granules.[2] Prior to the addition of **1,**

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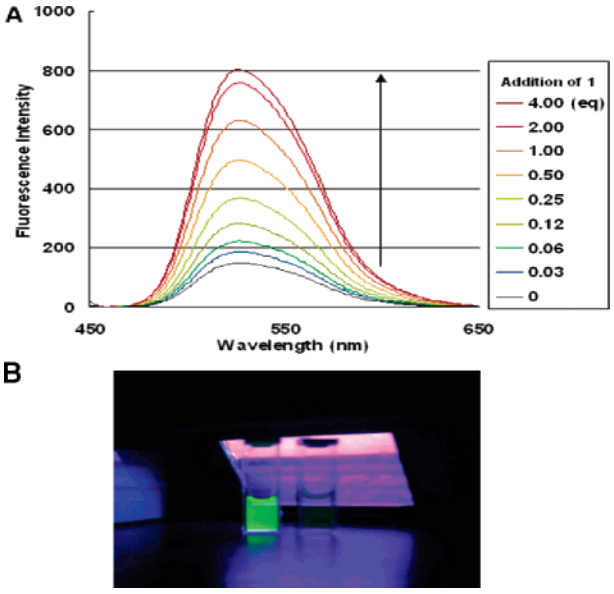

Figure 2. (A) Enhanced fluorescence of FAD (50 μM) upon addition of **1** (excitation, 445 nm; emission, 525 nm); (B) (left) increased FAD (10 μM) fluorescence upon addition of 2 equiv of **1**; (right) only FAD fluorescence (10 μM) under 365 nm UV lamp.

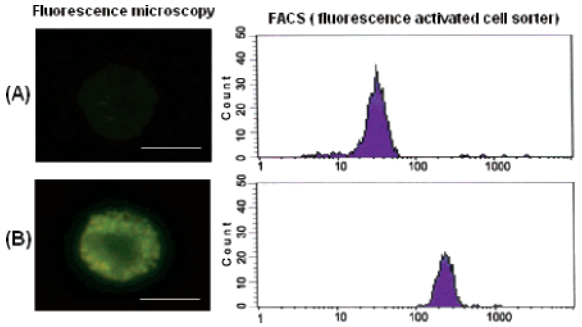

Figure 3. (A) Natural autofluorescence of eosinophils before incubation with **1**; (B) enhanced autofluorescence of eosinophils after 15 min of incubation with **1** (100 μM) in saline (150 mM NaCl, pH 7.4). The *x*-axis in FACS shows the fluorescence intensity of each eosinophil cell and the *y*-axis in FACS shows the eosinophil count. After incubation with **1**, the eosinophil distribution shifted to the stronger fluorescence intensity region. The cells were fixed and permeabilized prior to addition of **1**. Argon-ion laser (488 nm) was used for fluorescence excitation of cell and 530 ($\pm$10) nm emission filter was used in FACS. Scale bar = 10 μm.

eosinophils showed weak autofluorescence, and after incubation with **1** its granules showed bright greenish fluorescence (Figure 3). The enhanced autofluorescence of eosinophils was also detected by the fluorescence-activated cell sorter (FACS). The increased autofluorescence of the eosinophils due to addition of **1** can be used in the flow cytometry for eosinophil count;[11] this is crucial to immunological diagnosis for eosinophilia,[11a] an abnormal accumulation of eosinophils in blood or tissue, which occurs in certain disease states. The control experiment using **2** showed almost no enhancement of autofluorescence (see the SI). This is in good agreement with little fluorescence change of FAD upon addition of **2** as shown in Figure 4.

In summary, we have developed a novel fluorescent chemosensor that uses the Zn^{2+}-DPA complex, which selectively targets FAD

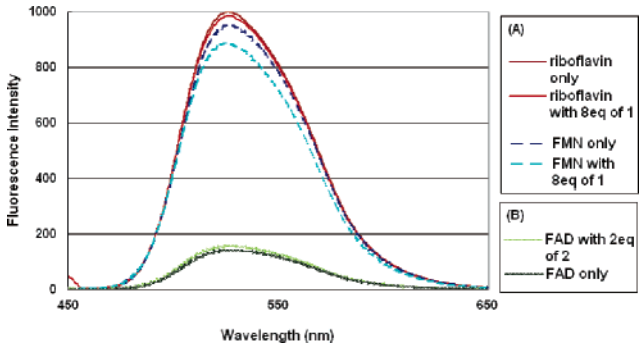

Figure 4. (A) Fluorescence changes of riboflavin (50 μM) and FMN (50 μM) with addition of **1** (excitation, 445 nm; emission, 525 nm); (B) fluorescence changes of FAD (50 μM) with **2** (excitation, 445 nm; emission, 525 nm).

from among other flavins in water. The enhancement of fluorescence emission of FAD was caused by a change in the intramolecular stacked conformation of FAD in water upon binding of the phosphodiester group of FAD to **1**. This probe was used to detect FAD in eosinophils by fluorescence microscopy and FACS.

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Supporting Information Available: Synthesis and results supporting the binding mode between **1** and FAD; complete procedures of cell study and results with neutrophils. This material is available free of charge via the Internet at http://pubs.acs.org.

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