Time-resolved Fluorescent Detection for Glucose Using a Complex of Luminescent Layered Titanates and Enzymes

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Luminescent europium-doped layered titanates (Eu-TiOx) were synthesized and complexed with horseradish peroxidase (HRP) and glucose oxidase (GOx). The emission of a resultant Eu-TiOx/HRP/GOx complex decreased upon the addition of glucose in the presence of guaiacol. The emission decrease was dependent on the concentrations of glucose, and the detection limit for glucose was 3.1 μ M. The proposed system would be promising as a new detection method for glucose.

Keywords Time-resolved fluorescent detection, glucose, layered titanates, horseradish peroxidase, glucose oxidase

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Detection methods for biomolecules are important as foundational techniques in biological and medical fields. Generally, fluorescent detection is practically simple, and is thus widely used for various kinds of biomolecules, as well as colorimetric detection. Fluorescent methods have higher sensitivity than colorimetric methods, and are applicable to visualizing a target analyte in cells by a fluorescent microscope.¹ Owing to these advantages, a variety of fluorescent methods have been reported for the detection of biomolecules.²⁻⁴

Inorganic layered materials are two-dimensional crystals that are utilized as photocatalysts,^{5,6} ion exchangers^{7,8} and a support in drug-delivery systems.⁹ The materials can be chemically exfoliated, and the resultant sheet-like crystallites (inorganic nanosheets) are restacked by intercalating different guests.¹⁰ By using this mechanism, various nanohybrids can be prepared.¹¹⁻¹⁴ Our group has reported on hybrid materials composed of layered titanates and peroxidase enzyme.¹⁵⁻¹⁸ By using lanthanides, luminescent properties can be added to thin layered materials.¹⁹⁻²¹ Although inorganic nanosheets are unique and attractive materials in this way, applications of the materials in analytical chemistry are very few at this stage, compared with inorganic semiconductor nanoparticles (quantum dots).²²⁻²⁷

In this study, fluorescent europium-doped titanate nanosheets (Eu-TiOx) were synthesized, and complexed with horseradish peroxidase (HRP) and glucose oxidase (GOx). The resultant complex (Eu-TiOx/HRP/GOx) was applied to the detection of glucose based on time-resolved fluorescent detection.

The synthesis of Eu-TiOx was performed according to a method reported by Ohya *et al.*²⁸ with minor modifications. Briefly, liquid titanium tetraisopropoxide (TTIP) was mixed with an ethanol solution of EuCl₃ (5 mol% for Eu to total metal content), and the mixed solution was then added to an aqueous solution of tetrabutylammonium hydroxide (TBAOH). The resultant solution was aged at 333 K for 2 h with gentle shaking,

and the obtained colorless and transparent colloidal solution was dialyzed by a membrane filter to reduce the pH of the solution. The Eu-TiOx (5 mM) after dialysis was mixed with HRP (0.25 mg/mL) and GOx (0.25 mg/mL) in 0.2 M acetate buffer (pH 4) for inducing complex formation through electrostatic interactions, and a resultant suspended solution was centrifuged. After removing the supernatant of the solution, the Eu-TiOx/HRP/GOx complex was obtained. The ratios of complex formation for HRP and GOx, estimated from absorbance measurements, are 98 and 96%, respectively.

Figure 1a shows the photoluminescence spectra of Eu-TiOx. The emission spectrum was obtained under excitation at 395 nm. The excitation peak at 395 nm would be assigned to an energy transfer from TiOx to Eu³⁺ ions.²¹ The emission peaks at 593 nm and 617 nm were assigned to ⁵D₀→⁷F₁ and ⁵D₀→⁷F₂ transitions of Eu³⁺, respectively.²¹ This result indicates that luminescent titanate nanosheets were successfully synthesized by the facile hydrolysis reaction. Figure 1b shows X-ray diffraction (XRD) patterns of the Eu-TiOx and Eu-TiOx/HRP/GOx complex. The peaks attributed to the (200) and (400) planes of the tetratitanate (Ti₄O₉²⁻) appeared at around 2 θ = 4.4° and 9.3° for Eu-TiOx, which means that Eu-TiOx nanosheets were stacked regularly with a certain space of intercalating TBA⁺ ion. On the contrary, no significant peak was observed for the Eu-TiOx/HRP/GOx complex. This result indicates that Eu-TiOx nanosheets in the Eu-TiOx/HRP/GOx complex exist irregularly, probably due to the small size of Eu-TiOx (3 nm, determined by dynamic light scattering (DLS) measurement).

The Eu-TiOx/HRP/GOx complex was then applied to the detection of glucose. In general, time-resolved fluorescent detection using lanthanides improves the detection limit because of the elimination of autofluorescence from biological samples.²⁹⁻³¹ Glucose was added to the Eu-TiOx/HRP/GOx complex in the presence of guaiacol, and the time-resolved fluorescent detection was performed using a microplate reader (SpectraMax™ i3x, Molecular devices) with a TRF cartridge (excitation wavelength, 370 nm; emission wavelength, 616 nm;

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Fig. 1 (a) Excitation and emission spectra of Eu-TiOx. The spectra were obtained by a spectrophotofluorometer (F-7000, Hitachi) using a phosphorescence-measurement mode ($\lambda_{\text{ex}} = 395 \text{ nm}$; $\lambda_{\text{em}} = 617 \text{ nm}$). (b) XRD patterns of Eu-TiOx and Eu-TiOx/HRP/GOx complex.

integration time, 1.89 ms; excitation time, 0.1 ms; number of pulses, 45; meas. delay, 0.01 ms; 96-well plate). Since luminescence lifetime for Eu^{3+} -intercalated layered titanium oxide in H_2O is reported to be *ca.* 0.1 – 0.3 ms,²¹ the condition for the time-resolved fluorescent detection is appropriate. Figure 2a shows an image of Eu-TiOx/HRP/GOx complexes in 96-well plate obtained by a transmitted light microscope. As can be seen from the figure, translucent particles were observed. Figure 3a shows changes in the emission intensity of the Eu-TiOx/HRP/GOx complex at 616 nm after the addition of glucose in the presence of guaiacol. The emission intensity gradually decreased upon the addition of glucose. Figure 2b shows images of the Eu-TiOx/HRP/GOx complex in 96-well plate after the addition of glucose in the presence of guaiacol. As can be seen from the figure, the Eu-TiOx/HRP/GOx complex was stained with brown. From these results, the decrease in the emission intensity in Fig. 3a would have been induced by the following steps (Scheme 1): (i) GOx in the Eu-TiOx/HRP/GOx complex produces hydrogen peroxides from added glucose, (ii) HRP in the Eu-TiOx/HRP/GOx complex proceeds the chromogenic reaction using the hydrogen peroxides and guaiacol in the solution and generates brown oxidation products of guaiacol, (iii) the brown products were adsorbed to the surface of the Eu-TiOx/HRP/GOx complex, probably due to hydrophobic interactions, and decrease in the emission intensity. As for the last step, the following two factors would cause the decrease in the emission intensity. The first is that the excitation

Fig. 2 Images obtained by a transmitted light microscope (Eu-TiOx/HRP/GOx complex, 625 μM Eu-TiOx containing 31 $\mu\text{g/mL}$ HRP and 30 $\mu\text{g/mL}$ GOx; scale bar, 50 μm). (a) Eu-TiOx/HRP/GOx complex. (b) Eu-TiOx/HRP/GOx complex after the addition of glucose (1000 μM) in the presence of guaiacol (2.5 mM). Incubation time was 90 min.

light at 370 nm would be partially absorbed by the brown products having broad absorption bands (330 – 800 nm). The second is that energy transfer would occur from the Eu-TiOx/HRP/GOx complex emitting at 616 nm to the adsorbed brown products.

Glucose with various concentrations (5, 50, 500, 1000 μM) was then added to the Eu-TiOx/HRP/GOx complex in the presence of guaiacol. Figure 3b shows the calibration curve for glucose based on time-resolved fluorescent detection. As can be seen from the figure, the emission intensity decreased with an increase in the concentration of glucose. The detection limit for glucose, calculated by $3\sigma/\text{slope}$ (σ : standard deviation of the blank signals), was 3.1 μM .

In conclusion, we studied the sensing of glucose based on time-resolved fluorescent detection using the Eu-TiOx/HRP/GOx complex. The proposed technique shows great promise as a new detection method for glucose with facility and high sensitivity. In addition, the method can be expanded to the sensing of other biomolecules by changing the combination of coupled enzymes. This study would be significant for new applications of inorganic nanosheets for analytical chemistry.

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Scheme 1 Schematic illustration of the detection mechanism for glucose in this study.

Fig. 3 Results of time-resolved fluorescent detection (Eu-TiOx/HRP/GOx complex, 625 μM Eu-TiOx containing 31 $\mu\text{g/mL}$ HRP and 30 $\mu\text{g/mL}$ GOx; 2.5 mM guaiacol). (a) Emission intensity of Eu-TiOx/HRP/GOx complex at 616 nm after the addition of glucose (1000 μM) in the presence of guaiacol. (b) Calibration curve for glucose (5, 50, 500, 1000 μM glucose; $n = 5$). Incubation time was 15 min.

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