学 位 論 文 内 容 の 要 旨/Dissertation Abstract

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学 位 論 文 題 名/Title of Dissertation

Development of solvatochromic fluorophores incorporating boron complex and application for biothiols detection in live-cell imaging

（ホウ素複合体を含むソルバトクロミック蛍光色素の開発と生体内細胞イメージングにおける生体チオール検出への応用）

Fluorescent probes have revolutionized biological research, enabling real-time visualization and tracking of molecular events within living cells. Among various applications, detecting biothiols such as glutathione (GSH), cysteine (Cys), and homocysteine (Hcy) is of significant interest due to their critical roles in maintaining cellular redox balance and protecting cells from oxidative damage. Their dysregulation is linked to multiple diseases, including cancer and neurodegenerative disorders. Abnormal biothiol levels are associated with diseases like cancer and neurodegenerative disorders. However, traditional fluorescent probes face challenges such as low signal resolution due to small Stokes shifts (the difference between excitation and emission wavelengths) and strong fluorescence even in the absence of biothiols. This doctoral dissertation has addressed these issues by designing advanced solvatochromic fluorophores with large Stokes shifts, enhanced sensitivity, and selectivity for biothiol detection, particularly in live-cell imaging.

At Yamada Laboratory, research on fluorescent solvatochromic dyes based on thiophene had been conducted, and it had been found that incorporating suitable electron-donating and electron-accepting moieties at the α-positions of thiophene can produce highly luminous fluorescent dyes. Among them, it is particularly understood that protonated or methylated pyridinium derivatives have absorption and fluorescence bands at longer wavelengths. Therefore, I considered that the cyanine structure, which is defined as a conjugated system between two nitrogen atoms, might be useful for the design of the target dye. However, since methylated pyridinium derivatives have low water solubility and are unstable, I have designed the boron complexes.

The *tert*-butoxycarbonyl (Boc) group, piperazine, phenyl group, thiophene pyridine, and boron directly connected molecule **BT-Boc**, a cyanine-based fluorophore precursor functionalized with a boron complex, tris(pentafluorophenyl)borane (BCF) has been designed and synthesized. This dye has been synthesized by Suzuki-Miyaura cross-coupling of Boc-protected phenylpiperazine as the electron-donating group, thiophene as the aromatic ring, and pyridine as the electron-accepting group, and complexation of BCF with the pyridine. The introduction of the BCF group enhanced the photophysical properties of **BT-Boc**, including red-shifted absorption and emission wavelengths, as well as an increased Stokes shift. Spectroscopic studies revealed that BT-Boc exhibited a maximum absorption red-shift of approximately 70 nm and an emission wavelength red-shift of 120 nm compared to its precursor *tert*-butyl 4-(4-(5-(pyridin-4-yl)thiophen-2-yl)phenyl)piperazine-1-carboxylate (**PT-Boc**). Additionally, **BT-Boc** demonstrated a significant Stokes shift of up to 200 nm in high-polarity solvents, highlighting the role of BCF coordination in enhancing fluorescence

properties. In addition, unlike methylated pyridinium derivatives, it was also found not to form micelle-like aggregates in water and not to self-quench.

To achieve biothiol specificity, a target probe relies on photoinduced electron transfer (PET) mechanisms to achieve fluorescence activation. The PET probes are designed to exhibit fluorescence quenching in their inactive state. Upon reacting with biothiols, the quenching is reversed, resulting in a fluorescence signal. This binary "off-on" mechanism ensures high selectivity and sensitivity. A 2,4-dinitrobenzenesulfonyl (DNBS) group reacts specifically with thiol groups (-SH) in biothiols, releasing the fluorophore and restoring fluorescence. This specificity enables accurate detection of biothiol concentrations in complex biological environments. To achieve the biothiol specificity, I have further modified of **BT-Boc** into **BT-DNBS** by deprotection and introduction of DNBS group.

BT-DNBS was rigorously tested *in vitro* for its ability to detect biothiols. To optimize the experimental conditions for biothiol detection, the fluorescence intensity of **BT-DNBS** (10 μM) was evaluated in different DMSO/PBS ratios. I found that a 9:1 ratio of DMSO to PBS was optimal. In this solvent, when **BT-DNBS** was reacted with 100 mm GSH, Cys, and Hcy, respectively, the fluorescence intensity was 80- , 40-, and 40-fold higher, respectively. **BT-DNBS** is strongly quenched by the PET mechanism but exhibited a strong fluorescent response to GSH and Cys by canceling the PET mechanism, with detection limits suitable for live-cell applications. And it showed minimal interference from other biological molecules, such as amino acids and metal ions, underscoring its specificity for biothiols. Furthermore, its fluorescent response was rapid and sustained compared with the reported DNBS-type fluorescent probes, making it ideal for real-time imaging. These experimental results suggested that **BT-DNBS** has good performance for detecting biothiols *in vivo*.

Using A431 cells as the model, I evaluated the biocompatibility and imaging performance of **BT-DNBS** for intracellular biothiol detection. Cytotoxicity was assessed using the cell-counting-kit-8 (CCK-8) assay, which measures cell viability based on the reduction of WST-8 to a water-soluble formazan dye by cellular dehydrogenases. The results demonstrated low cytotoxicity at the tested concentrations, confirming the probe's safety for live-cell imaging applications. Subsequently, fluorescence imaging experiments revealed time-dependent fluorescence enhancement upon incubation with **BT-DNBS**, demonstrating the probe's ability to detect intracellular biothiols. A431 cells, widely used in fluorescence imaging studies due to their well-characterized properties and suitability for evaluating intracellular probes, were chosen for this investigation. Pre-treatment with N-ethylmaleimide (NEM), a thiol-blocking agent, significantly suppressed fluorescence, validating the probe's biothiol specificity. Confocal laser scanning microscopy further revealed bright intracellular fluorescence after staining, indicating effective cellular uptake and reaction with intracellular thiols. These results underscore the potential of **BT-DNBS** as a biocompatible and sensitive fluorescent probe for intracellular biothiol detection in biological research.

This doctoral dissertation successfully has developed and validated a novel fluorescent probe for biothiol detection, incorporating innovative design principles to overcome limitations of existing technologies. The **BT-DNBS** probe, with its large Stokes shift, high photostability, and excellent specificity, represents a significant advancement in the field of live-cell imaging. Its potential applications span basic biological research, disease diagnostics, and therapeutic development, underscoring its value as a versatile tool for studying cellular processes and pathological mechanisms.

Future work may focus on further optimizing the probe for *in vivo* applications, exploring its use in other redox-related biomolecules, and integrating it into multimodal imaging platforms to expand its utility in biomedical research.