



รายงานวิจัยฉบับสมบูรณ์

โครงการ การสังเคราะห์เซนเซอร์ที่มีไดฟลูออโรโบราอะเซตติลอะซิโตนเคอร์คูมินและ/หรือแอน
ทราควิโนนอิมิดาโซลเป็นหน่วยพื้นฐานและการศึกษาสมบัติการเป็นเซนเซอร์กับนิวคลีโอไทด์

โดย นางสาวบุษยรัตน์ ธรรมพัฒน์กิจ

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นางสาวบุษยรัตน์ ธรรมพัฒน์กิจ

จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยทบวงมหาวิทยาลัย และสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย ทบวงฯ และสกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

ได้สังเคราะห์เซ็นเซอร์ที่เป็นอนุพันธ์ของแอนทราควิโนนอิมิดาโซลและกรดโบโรนิก และนำอนุพันธ์ของแอนทราควิโนนและกรดโบโรนิก (OB และ PB) มาศึกษาสมบัติในการเป็นเซ็นเซอร์ในการตรวจวัดน้ำตาลกลูโคสในระบบการตรวจวัดด้วยเอนไซม์ พบว่าเซ็นเซอร์นี้มีความจำเพาะเจาะจงกับน้ำตาลกลูโคสเท่านั้น และได้คำนวณขีดจำกัดต่ำสุดในการตรวจวัดน้ำตาลในช่วงความเข้มข้นเท่ากับ 0.08-0.42 มิลลิโมลาร์ ได้ขีดจำกัดต่ำสุดเท่ากับ 0.0114 มิลลิโมลาร์

สังเคราะห์เซ็นเซอร์ที่มีอนุพันธ์ของฟลูออเรสซินและกรดโบโรนิก **F-oBOH** **F-mBOH** และ **F-pBOH** เพื่อใช้ตรวจวัดคอปเปอร์และไซยาไนด์ไอออน พบว่าเซ็นเซอร์ทั้งสามตัวเกิดปฏิกิริยาไฮโดรไลซิสได้เมื่ออยู่ในน้ำ จึงใช้ coordination nanoparticles เพื่อป้องกันเซ็นเซอร์จากการไฮโดรไลซิสพบว่า **F-oBOH-AMP/Gd³⁺ CNPs** คอปเปอร์ (II) มีความจำเพาะเจาะจงกับไซยาไนด์ไอออนเท่านั้น และมีค่าคงที่ในการเกิดสารประกอบเชิงซ้อนระหว่าง **F-oBOH-AMP/Gd³⁺ CNPs** คอปเปอร์(II) และไซยาไนด์ เท่ากับ 3.97 ขีดความสามารถในการตรวจวัดเท่ากับ 4.03 μM นอกจากนี้พบว่าแอนไอออนชนิดต่าง ๆ สามารถรบกวนการตรวจวัดไซยาไนด์ไอออนน้อยมาก

ได้สังเคราะห์ฟลูออเรสเซนซ์เซ็นเซอร์ (**L1**) ที่มีไคฟลูออโรเคอร์คิวมินและไดฟีโคลิเวมีนเป็นองค์ประกอบ จากนั้นศึกษาการเกิดสารประกอบเชิงซ้อนของเซ็นเซอร์ **L1** กับ Zn^{2+} และ Cu^{2+} ด้วยเทคนิคยูวีวิสิเบิลและฟลูออเรสเซนซ์ พบว่าเกิดสารประกอบเชิงซ้อนของเซ็นเซอร์ต่อ Zn^{2+} และ Cu^{2+} ในอัตราส่วน 1:2 และทำให้เกิดการเคลื่อนของค่าการดูดกลืนแสงสูงสุดไปที่ความยาวคลื่นต่ำลง และส่งผลให้ความเข้มของสเปกตรัมการคายแสงที่ 587 nm ลดลง และได้สังเคราะห์เซ็นเซอร์ชนิดใหม่ที่ประกอบด้วยไพรีนและสารประกอบเชิงซ้อนไดฟีโคลิเวมีน (**L2**) ที่จับกับสังกะสีเพื่อตรวจวัดนิวคลีโอไทด์ในน้ำ พบว่า เซ็นเซอร์ **L2•Zn** มีความจำเพาะเจาะจงกับไพโรฟอสเฟตในน้ำ แต่ในระบบตัวทำละลายอินทรีย์ DMSO:HEPES (0.01 M, pH 7.4 90:10 v/v) พบว่า สามารถจับกับนิวคลีโอไทด์ที่มีหมู่ฟอสเฟต 3 หมู่ได้ดี ได้แก่ ATP และ UTP โดยทำให้ความเข้มของสเปกตรัมการคายแสงเพิ่มขึ้น เมื่อนำการวิเคราะห์องค์ประกอบหลัก (Principal Component Analysis) มาใช้ในการแยกความสามารถในการจับกับนิวคลีโอไทด์ทั้ง 10 ชนิดในตัวทำละลายทั้งสองระบบ วิธีการนี้สามารถแยกสารตัวอย่างได้ด้วยความแม่นยำ 88 เปอร์เซ็นต์ นอกจากนี้ยังสามารถประยุกต์ใช้ในการหาอัตราส่วนระหว่างไพโรฟอสเฟตกับ ATP ในกระบวนการ ATP hydrolysis ได้อีกด้วย

สำหรับการจำแนกประเภทของนิวคลีโอไทด์โดยใช้การวิเคราะห์องค์ประกอบหลัก โมเลกุลเซ็นเซอร์ที่ใช้ในการศึกษานี้จะประกอบด้วยอนุพันธ์ของไพรีนและคูมาริน (**Pydpa** และ **Es**) เพื่อใช้ในการแยกประเภทของนิวคลีโอไทด์ ในขั้นต้นได้เตรียมสารประกอบเชิงซ้อนของเซ็นเซอร์โมเลกุล **Pydpa-Zn**, **Pydpa-Cu**, **Es-Zn** และ **Es-Cu** เพื่อใช้จับกับ นิวคลีโอไทด์ ซึ่งหมู่ฟอสเฟตของนิวคลีโอไทด์ สามารถเกิดอันตรกิริยาทางไฟฟ้าสถิตกับสารประกอบเชิงซ้อนของเซ็นเซอร์โมเลกุลได้ จากการศึกษาความสามารถในการเกิดสารประกอบเชิงซ้อน โดยใช้เทคนิคฟลูออเรสเซนซ์สเปกโตรโฟโตเมตรี พบว่า **Pydpa-Zn**, **Es-Zn** และ **Es-Cu** มีความจำเพาะต่อ PPi สูง ได้ค่าคงที่การจับ ($\log K$) ของ **Pydpa-Zn**, **Es-Zn** และ **Es-Cu** กับ PPi เท่ากับ 8.83, 5.23 และ 4.64 ตามลำดับ นอกจากนี้ผู้วิจัยได้นำการวิเคราะห์องค์ประกอบหลัก มาใช้แยกประเภทของนิวคลีโอไทด์โดยการสร้างเป็นเซ็นเซอร์อาร์เรย์ (การรวมกันของข้อมูลแต่ละเซ็นเซอร์) และสร้างเป็นเซ็นเซอร์ใหม่ (การรวมกันของเซ็นเซอร์โมเลกุล) ซึ่งเป็นที่น่าสนใจว่าข้อมูลที่ได้จากการรวมกันของเซ็นเซอร์ **Pydpa-Zn+Es-Cu** จะแสดงการจำแนกกลุ่มของนิวคลีโอไทด์ PPi , ATP, UTP, ADP, UDP และ GDP ได้อย่างชัดเจน และเปอร์เซ็นต์ความถูกต้องของการทำนายเชิงกลุ่มเป็น 92%

Abstract

The anthraquinone derivatives containing boronic acid or glycoside as an active site were synthesized and characterized by spectroscopy. We have developed the boronic anthraquinone based sensors (**OB** and **PB**) for glucose sensing by the enzymatic probe system with glucose oxidase. The advantage of this sensor is that it can detect glucose specifically. The limit of detection (LOD) of glucose for **OB** evaluated in the range of 0.08-0.42 mM of glucose by the spectrofluorometry was 0.0114 mM.

A new sensors containing fluorescein and boronic acid moieties, **F-oBOH**, **F-mBOH**, and **F-pBOH**, for detection of copper and cyanide ions. In addition, we discovered that sensors were hydrolyzed by water. Therefore, we designed the new strategy to prevent the hydrolysis approach by coordination nanoparticles network (**F-oBOH-AMP/Gd³⁺ CNPs**). The log K value of **F-oBOH-AMP/Gd³⁺ CNPs** $\subset$ copper (II) with cyanide ion was 3.97. The detection limit of **F-oBOH-AMP/Gd³⁺ CNPs** $\subset$ copper(II) with cyanide anion was 4.03 μ M. Furthermore, they possess a high selectivity for cyanide ion compared to other competitive anions.

A new fluorescence sensor (**L1**), based on BF₂-Curcumin and dpa (bis (2-pyridylmethyl)amine) for detection of Zn²⁺ and Cu²⁺ ions was synthesized. The sensor demonstrates the blue-shifted absorption band and the fluorescence quenching at 587 nm in acetonitrile after adding both metal ions. Moreover, we developed the chemosensor containing pyrene and dpa-Zn complex (**L2•Zn**) for detection of nucleotide in aqueous solution. This sensor has a high selectivity for PPI in aqueous solution. In contrast for the case of DMSO:HEPES (0.01 M, pH 7.4 90:10 v/v), **L2•Zn** selectively binds to nucleoside polyphosphates such as ATP and UTP with a large fluorescence enhancement. The Principal Component Analysis (PCA) was used to discriminate between 10 nucleotides by two solvent systems. This approach was found to identify 10 analytes with 88% accuracy. To illustrate the utility of this approach for ATP hydrolysis application, the ratio between the product of reaction (PPI) and the reactant (ATP) can be used in quantitative identification of the PPI and ATP ratios.

For nucleotides discrimination using Principal Component Analysis (PCA), the sensory molecules containing pyrene and coumarin derivatives (**Pydpa** and **Es**) were used for discrimination of various nucleotides in aqueous solution. Initially, the complexation of **Pydpa-Zn**, **Pydpa-Cu**, **Es-Zn**, and **Es-Cu** were prepared as the sensory molecules to recognize the phosphate anion based nucleotides undergone the electrostatic interaction. The complexation studies of these sensors were carried out by fluorescence spectrophotometry. It was found that **Pydpa-Zn**, **Es-Zn**, and **Es-Cu** showed a high selectivity to PPI with the log K values of 8.83, 5.23 and 4.64, respectively. In addition, the PCA method was applied to classify the types of nucleotides by creation of the sensor arrays (the combinatorial data) and the mixed sensors (the combinatorial sensors). Interestingly, the combinatorial sensors of **Pydpa-Zn+Es-Cu** showed a highly potential discrimination of PPI, ATP, UTP, ADP, UDP and GDP with 92% classification accuracy.

Executive Summary

1. ความสำคัญและที่มาของปัญหา

ในปัจจุบันนี้มีนักวิจัยหลายสาขาที่ศึกษาวิจัยเกี่ยวกับเคมีเชิงซูพราโมเลกุล (Supramolecular Chemistry) กันอย่างแพร่หลาย ซึ่งความรู้เคมีด้านนี้ สามารถนำไปประยุกต์ใช้กับงานได้หลายประเภท อาทิเช่น การสังเคราะห์โมเลกุลที่เป็นเซนเซอร์ทางเคมี โมเลกุลที่มีความสามารถในการเป็นตัวเร่งปฏิกิริยา การประยุกต์โมเลกุลที่สังเคราะห์ได้ไปใช้เป็นไบโอเซนเซอร์ (biosensor) การนำไปใช้ในด้านเภสัชศาสตร์และการแพทย์ แต่ถ้ากล่าวถึงงานทางด้านเคมี จะนิยมทำการศึกษาวิจัยเกี่ยวกับเซนเซอร์ทางเคมีโดยการสังเคราะห์โมเลกุลใหม่ๆ ที่มีความสามารถในการจับกับแคทไอออน แอนไอออน และโมเลกุลอินทรีย์ที่เป็นกลางได้อย่างจำเพาะเจาะจง แต่อย่างไรก็ตามยังมีปัญหาใหญ่ที่เกิดขึ้นคือถ้าหากโมเลกุลโฮสต์เกิดอันตรกิริยากับเกสต์แบบนอนโควาเลนต์ เช่น พันธะไฮโดรเจน จะทำให้โมเลกุลของน้ำที่อยู่ในระบบแข่งขันกับเกสต์ในการเกิดอันตรกิริยากับโฮสต์ ดังนั้นนักเคมีจึงได้ออกแบบโครงสร้างโมเลกุลเซนเซอร์ให้บริเวณที่เป็น binding site สามารถเกิดพันธะโควาเลนต์กับเกสต์ได้ พันธะโควาเลนต์จะเสถียรกว่าพันธะไฮโดรเจนและจะช่วยแก้ปัญหาเรื่องการแข่งขันของเกสต์กับน้ำได้

ได้มีกลุ่มวิจัยหลายกลุ่มที่ศึกษาเกี่ยวกับโมเลกุลโฮสต์ที่มีอนุพันธ์ของกรดโบโรนิก (boronic acid) เพื่อใช้ตรวจจับกับสารประกอบจำพวกแซคคาไรด์ (saccharide) หรือสารประกอบชนิดอื่น ๆ ที่มีหมู่ไดออล อาทิเช่น น้ำตาลกลูโคส ฟรุคโทส กาแลคโทส เป็นต้น ซึ่งกรดโบโรนิกนั้นมีความสามารถในการเกิดปฏิกิริยากับสารประกอบแซคคาไรด์อย่างจำเพาะเจาะจง โดยปฏิกิริยาที่เกิดขึ้นเป็นปฏิกิริยาโควาเลนต์ระหว่างกรดโบโรนิกและหมู่ไดออล (diol) ที่มีอยู่ในโมเลกุล ซึ่งปฏิกิริยาที่เกิดขึ้นเป็นชนิดผันกลับได้ (reversible formation)

นอกจากนี้จากการศึกษาพบว่า รีเซปเตอร์ที่มีอนุพันธ์ของกรดโบโรนิกสามารถเกิดสารประกอบเชิงซ้อนที่เสถียรได้กับสารประเภทลิวอิสเบส (Lewis Base) เช่น ฟอสเฟต ซิเตรต และอิมิดาโซล ดังนั้น จึงสามารถนำสารประกอบโบโรนิกมาศึกษาการจับกับโมเลกุลอินทรีย์ที่มีฟอสเฟตเป็นองค์ประกอบได้ ในการศึกษาการจับกันของโมเลกุลโฮสต์กับเกสต์นั้นได้นำเทคนิคมากมายมาใช้ในการวิเคราะห์สารที่สนใจ เช่น เทคนิคทางแสง ได้แก่ ยู-วี-วิสิเบิล และฟลูออเรสเซนซ์ เป็นต้น และเทคนิคทางเคมีไฟฟ้า เทคนิคเหล่านี้เป็นที่นิยมในงานด้านเคมีเชิงซูพราโมเลกุล เพราะเมื่อมีการจับกันของโฮสต์ที่ออกแบบมาให้เหมาะสมกับเกสต์แล้ว ใช้เทคนิคเหล่านี้ในการตรวจวัดจะเห็นการเปลี่ยนแปลงอย่างชัดเจน ซึ่งโฮสต์นั้นต้องประกอบด้วยหน่วยให้สัญญาณ (signaling unit) และหน่วยจับ (binding unit) หน่วยให้สัญญาณนั้น พบว่าโมเลกุลที่มีหมู่ฟลูออโรฟอร์เป็นองค์ประกอบนั้นจะทำให้ sensitivity สูงขึ้น ซึ่งหมายความว่าสามารถตรวจจับเกสต์ในปริมาณน้อยได้ และนอกจากนี้ถ้ารีเซปเตอร์เป็นสารที่มีสีและเมื่อมีการจับกับเกสต์แล้วจะให้สี

เปลี่ยนแปลงอย่างชัดเจนก็จะทำให้ง่ายต่อการวิเคราะห์ ดังนั้นในปัจจุบันนี้จึงมีการใช้สารสี้อมมาเป็นหน่วยให้สัญญาณ

ในงานวิจัยที่เสนอขอทุนสนับสนุนนี้เลือกใช้เคอร์คูมิน (curcumin) หรือ 1,7-บิส[4-ไฮดรอกซี-3-เมทออกซีฟีนิล]-1,6-เฮปตะไดเอน-3,5-ไดโอน (1,7-bis[4-hydroxyphenyl]-1,6-heptadiene-3,5-dione) ซึ่งเป็นสารสี้อมทางธรรมชาติที่มีสีเหลืองส้มมีอยู่มากหรือเป็นสารหลักในขมิ้นชันในวงศ์ ZINGIBERACEAE มาทำการดัดแปลงโครงสร้างให้มีหมู่โบโรนิกแอซิดเพื่อสร้างเป็นเซนเซอร์ชนิดใหม่สำหรับโมเลกุลและไอออนที่สำคัญในทางชีวภาพเช่น ATP และ ADP เป็นต้น นอกจากนี้ยังได้สังเคราะห์โมเลกุลเซนเซอร์ที่มีแอนทราควิโนนอิมิดาโซล (anthraquinoneimidazole) เป็นหน่วยพื้นฐานเพื่อใช้ในวัตถุประสงค์เดียวกันด้วย

2. วัตถุประสงค์ของโครงการ

1. สังเคราะห์ฟลูออเรสเซนส์เซนเซอร์ตัวใหม่โดยใช้นุพันธ์ของเคอร์คูมินที่มีโบรอนไดฟลูออไรด์และมีแอนทราควิโนนอิมิดาโซล เป็นส่วนให้สัญญาณและมีหมู่โบโรนิกและฟิโคลิดเป็นตัวจับนิวคลีโอไทด์
2. ศึกษาพฤติกรรมการดูดกลืนแสงและการคายแสงของโมเลกุลที่สังเคราะห์ได้
3. ศึกษาความสามารถในการเกิดสารประกอบเชิงซ้อนกับนิวคลีโอไทด์แต่ละชนิด เช่น ATP, ADP, AMP, UDP, UTP, CTP, UMP, และ GTP และศึกษาพฤติกรรมทางแสงเมื่อเกิดสารประกอบเชิงซ้อนกับเกสต์

3. ระเบียบวิธีการวิจัย

โครงการย่อยที่ 1: การสังเคราะห์สารที่มีแอนทราควิโนนอิมิดาโซล เป็นโมเลกุลพื้นฐานและศึกษาการเกิดสารประกอบเชิงซ้อนกับซิงค์แคปไทออนและนิวคลีโอไทด์ชนิดต่างๆ

- 1.1) ศึกษาค้นคว้าและรวบรวมข้อมูลที่เกี่ยวข้อง
- 1.2) สังเคราะห์เซนเซอร์โมเลกุลที่มีแอนทราควิโนนอิมิดาโซลเป็นโมเลกุลพื้นฐานและเป็นหน่วยให้สัญญาณฟลูออเรสเซนส์ และมีหน่วยโบโรนิกเป็นส่วนจับกับเกสต์; *o*-AQB, *p*-AQB และ *m*-AQB
- 1.3) พิสูจน์เอกลักษณ์ของสารที่สังเคราะห์ได้ ด้วยวิธีทางสเปกโทรสโกปี
- 1.4) หาสภาวะที่เหมาะสมที่ไม่ทำให้ Zn^{2+} ตกตะกอน และ ไดออกของหน่วยน้ำตาลในนิวคลีโอไทด์สามารถจับกับหมู่โบโรนิกได้ เช่น การหาค่า pK_a ของ หมู่โบโรนิก และ pH ที่เหมาะสมในการเป็นบัฟเฟอร์ของสารละลายน้ำ

- 1.5) ศึกษาการเกิดสารประกอบเชิงซ้อนของ $[\text{Zn}(o\text{-AQB})]^{2+}$, $[\text{Zn}(p\text{-AQB})]^{2+}$ และ $[\text{Zn}(m\text{-AQB})]^{2+}$ กับนิวคลีโอไทด์ชนิดต่างๆ ในสารละลายบัฟเฟอร์ และ หาค่าคงที่ของการเกิดสารประกอบเชิงซ้อนของ ด้วยเทคนิคฟลูออเรสเซนซ์ไทเทรชัน
- 1.6) พิสูจน์โครงสร้างของสารประกอบเชิงซ้อน ด้วยเทคนิคเอกซ์เรย์คริสตัลโลกราฟี (ถ้าสามารถตก single crystal ได้) หรือใช้เทคนิค 2D-NMR spectrometry

โครงการย่อยที่ 2: การสังเคราะห์สารที่มี difluoro-bora-acetylacetonate-curcumin และโบโรนิกแอซิดเป็นโมเลกุลพื้นฐานและศึกษาการเกิดสารประกอบเชิงซ้อนกับนิวคลีโอไทด์

- 2.1) สังเคราะห์เซนเซอร์โมเลกุลที่มี **BF₂-Curcumin** เป็นโมเลกุลพื้นฐานและเป็นหน่วยให้สัญญาณฟลูออเรสเซนซ์ และมีหน่วยโบโรนิกเป็นส่วนจับกับเกสต์; **BF₂-Cur-*p*-BOH** และ **BF₂-Cur-*m*-BOH**
- 2.2) พิสูจน์เอกลักษณ์ของสารที่สังเคราะห์ได้ ด้วยวิธีทางสเปกโทรสโกปี
- 2.3) หาค่า pK_a ของ หมู่โบโรนิกแอซิดในเซนเซอร์โมเลกุล และ pH ที่เหมาะสมในการเป็นบัฟเฟอร์ของสารละลายน้ำ
- 2.4) ศึกษาการเกิดสารประกอบเชิงซ้อนของ **BF₂-Cur-*p*-BOH** และ **BF₂-Cur-*m*-BOH** กับ นิวคลีโอไทด์ชนิดต่างๆ ในสารละลายบัฟเฟอร์ และ หาค่าคงที่ของการเกิดสารประกอบเชิงซ้อน ด้วยเทคนิคฟลูออเรสเซนซ์ไทเทรชัน
- 2.5) พิสูจน์โครงสร้างของสารประกอบเชิงซ้อน ด้วยเทคนิคเอกซ์เรย์คริสตัลโลกราฟี (ถ้าสามารถตก single crystal ได้) หรือใช้เทคนิค 2D-NMR spectrometry

โครงการย่อยที่ 3: การสังเคราะห์สารที่มี difluoro-bora-acetylacetonate-curcumin และฟิโคลิดเป็นโมเลกุลพื้นฐานและศึกษาการเกิดสารประกอบเชิงซ้อนกับนิวคลีโอไทด์

- 3.1) สังเคราะห์เซนเซอร์โมเลกุลที่มี **BF₂-Curcumin** เป็นโมเลกุลพื้นฐานและเป็นหน่วยให้สัญญาณฟลูออเรสเซนซ์และมีหน่วยฟิโคลิดหรือหน่วยโบโรนิกเป็นส่วนจับกับเกสต์
- 3.2) พิสูจน์เอกลักษณ์ของสารที่สังเคราะห์ได้ด้วยวิธีทางสเปกโทรสโกปี
- 3.3) หาค่า pK_a ของ หมู่โบโรนิก และ pH ที่เหมาะสมในการเป็นบัฟเฟอร์ของสารละลายน้ำ
- 3.4) ศึกษาการเกิดสารประกอบเชิงซ้อนของ $[\text{Zn}(\text{BF}_2\text{-Cur-BisPC})]^{2+}$, $[\text{Zn}(\text{BF}_2\text{-Cur-PC})]^{2+}$, $[\text{Zn}(\text{BF}_2\text{-Cur-*m*-BOH-PC})]^{2+}$ และ $[\text{Zn}(\text{BF}_2\text{-Cur-*p*-BOH-PC})]^{2+}$ กับ นิวคลีโอไทด์ชนิดต่างๆ ในสารละลายบัฟเฟอร์ และ หาค่าคงที่ของการเกิดสารประกอบเชิงซ้อนด้วยเทคนิคฟลูออเรสเซนซ์ไทเทรชัน
- 3.5) พิสูจน์โครงสร้างของสารประกอบเชิงซ้อน ด้วยเทคนิคเอกซ์เรย์คริสตัลโลกราฟี (ถ้าสามารถตก single crystal ได้) หรือใช้เทคนิค 2D-NMR spectrometry

จากการทดลองข้างต้นเมื่อทราบโมเลกุลเซนเซอร์ที่เกิดอันตรกิริยากับนิวคลีโอไทด์ได้ดีที่สุดแล้วจะหาสถานะและองค์ประกอบที่ดีที่สุดในการนำสารที่สังเคราะห์หรือดัดแปลงได้นำมาสร้างเป็นเซนเซอร์ที่ขึ้นรูปเป็นชิ้นงานสำหรับตรวจจับนิวคลีโอไทด์ แล้วนำไปทดลองใช้งานในระบบชีวภาพจริง

4. แผนการดำเนินงานตลอดโครงการ (2553-2555)

ตารางที่ 1 แสดงแผนงาน

กิจกรรม	ปีที่ 1						ปีที่ 2						ปีที่ 3					
	02	04	06	08	10	12	14	16	18	20	22	24	26	28	30	32	34	36
1: โครงการย่อยที่ 1																		
1.1 ศึกษาค้นคว้าและรวบรวมข้อมูลที่เกี่ยวข้อง	↔		↔					↔										
1.2 สังเคราะห์เซนเซอร์ <i>o</i> -AQB, <i>p</i> -AQB และ <i>m</i> -AQB	↔			↔														
1.3 พิสูจน์เอกลักษณ์ของสารที่สังเคราะห์ได้ ด้วยวิธีทางสเปกโทรสโกปี				↔														
1.4 หาสภาวะที่เหมาะสม เช่น การหาค่า pK_u ของหมู่ไบโรนิก และ pH ที่เหมาะสมในการเป็นบัฟเฟอร์ของสารละลายยา				↔														
1.5 เตรียมสารประกอบเชิงซ้อน Zn complexes							↔											
1.6 ศึกษาการเกิดสารประกอบเชิงซ้อนและหาค่าคงที่ของ Zn complex กับ นิวคลีโอไทด์ชนิดต่างๆ ในสารละลายบัฟเฟอร์ด้วยเทคนิคฟลูออเรสเซนซ์โพเทนเชียล							↔											

[illegible]

5. ชื่อเรื่องและชื่อวารสารที่คาดว่าจะตีพิมพ์ในวารสารวิชาการนานาชาติ

คาดว่าจะผลงาน 2 เรื่องตีพิมพ์ในวารสารต่างประเทศ เช่น Tetrahedron Letters (IF = 2.538) Tetrahedron (IF = 2.897) และ Journal of Organic Chemistry (3.952) โดยเรื่องที่จะตีพิมพ์ได้แก่

- 1) Nucleotide probes based on BF_2 -Curcumine and boronic acid in aqueous
- 2) Complexation studies of Zn-receptors containing BF_2 -Curcumine and picolyl with nucleotide in aqueous

6. รายชื่อนักศึกษา

1. นางสาว ศรกฤษณ์ มาบำรุง
2. นางสาว ศิรินันท์ กุลชาติ
3. นางสาว ญานิศ สวงนทรัพย์

7. งบประมาณโครงการ

หมวดงบประมาณ	ปีที่ 1	ปีที่ 2	ปีที่ 3	รวม
1. หมวดค่าตอบแทน	180,000.00	180,000.00	180,000.00	540,000.00
2. หมวดค่าวัสดุ				
● ค่าสารเคมี	167,000.00	157,000.00	151,000.00	475,000.00
- ตัวทำละลาย				
- ตัวทำละลายสำหรับ				
NMR	10,000.00	10,000.00	10,000.00	30,000.00
● เครื่องแก้ว	10,000.00	10,000.00	10,000.00	30,000.00
● ค่าวัสดุสิ้นเปลือง	3,000.00	3,000.00	4,000.00	10,000.00
● ค่าวัสดุสำนักงาน				
3. หมวดค่าใช้สอย				
● ค่าวิเคราะห์	15,000.00	25,000.00	25,000.00	65,000.00
● ค่าซ่อมเครื่องแก้ว	10,000.00	10,000.00	10,000.00	30,000.00
● ค่าถ่ายเอกสาร ค่าค้นคว้า	5,000.00	5,000.00	10,000.00	20,000.00
ข้อมูลและอื่นๆ ค่าจ้าง				
พิมพ์รายงาน ค่าน้ำและ				
ไฟฟ้า ค่าไปรษณีย์และ				
อื่นๆ				
รวมงบประมาณโครงการ	400,000.00	400,000.00	400,000.00	1,200,000.00

Introduction

Fluorescence spectroscopy techniques

Fluorescence spectroscopy, one of the most informative and sensitive analytical techniques, has played and continues to play key roles in modern research. Indeed, unraveling the inner workings of biomolecules, cells and organisms relied on the development of fluorescence-based tools. As many of the players in these sophisticated interactions and exceedingly complex systems are not inherently emissive, researchers have relied on synthesizing fluorescent analogs of the building blocks found in biological macromolecules. These are the constituents of the cell surface and cell membrane, as well as proteins and nucleic acids.

Any spectroscopy-based technique is associated with inherent sensitivity traits and time-scale features, which are dependent on the fundamental nature of the transitions involved. Optical excitation of a chromophore generates the Franck-Condon state extremely rapidly (within 10^{-15} s). The efficiency of this process is related to the chromophore's absorption cross-section (σ), which is proportional to its extinction coefficient (ϵ). Vibrational relaxation (within 10^{-12} - 10^{-10} s) quickly populates the lowest vibronic state of the chromophore's excited state (**Figure 1**) [1]. This relaxation process, generating the emissive state, accounts for the lower emission energy of a chromophore compared with its excitation energy (Stokes shift). Typical organic chromophores reside in their excited state for a period of $(0.5-20) \times 10^{-9}$ s. The excited state lifetime reflects the sum of the various radiative and nonradiative processes that the excited chromophore undergoes in decaying back to the ground state (τ_0). The fraction responsible for emitting a photon, or the fluorescence lifetime (τ), reflects the emission quantum yield of the chromophores ($Q = \Phi = \tau/\tau_0$). In some studies, the brightness ($\epsilon\Phi$) of a fluorophore is reported, which is the product of the molar absorptivity (ϵ) and the fluorescence quantum yield (Φ). This becomes useful when comparing the utility of two fluorophores with similar fluorescence quantum yields but very different molar absorptivities.

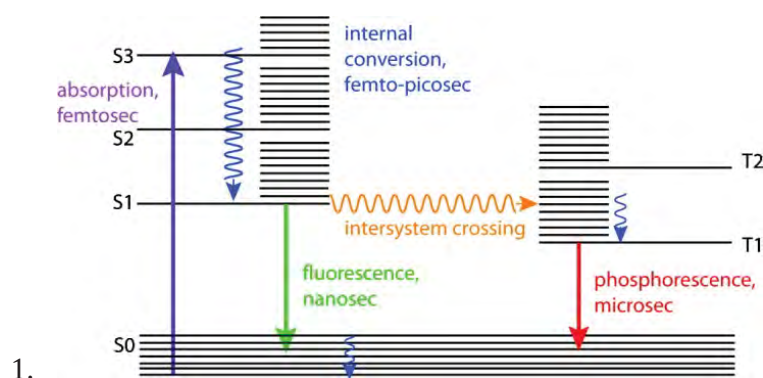


Figure 1 Jablonski diagram and a time scale of photophysical processes for organic molecules

Fluorescence based techniques are commonly appreciated for their versatility and sensitivity (up to a 1000-fold higher than absorption spectrophotometry). Creative probe design can provide chromophores with appropriate excitation and emission wavelengths, while minimizing interference by other emissive cellular constituents. Selective excitation coupled to the sensitivity of many chromophores to various environmental parameters (pH, polarity, viscosity, presence of quenchers, etc.) makes molecular fluorescence an extremely effective tool for *in vitro* biophysical and biochemical analyses, as well as *in vivo* cellular imaging, capable of providing spatial and temporal information [1-3].

Photophysics of fluorescent sensors

Photoinduced electron transfer (PeT)

In the simplest cases, emission of a photon, fluorescence, follows HOMO to LUMO excitation of an electron in a molecule. Where this emission is efficient, the molecule may be termed a fluorophore. Vibrational deactivation of the excited state prior to emission usually gives rise to a “Stokes shift” in that the wavelength of the emitted radiation is less than that of the exciting radiation [4]. Various other interactions may also modify the emission process, and these are of considerable importance in regard to analytical applications of fluorescence.

Thus, when a lone pair electron is located in an orbital of the fluorophore itself or an adjacent molecule and the energy of this orbital lies between those of the HOMO and LUMO, efficient electron transfer of one electron of the pair to the hole in the HOMO

created by light absorption may occur, followed by transfer of the initially excited electron to the lone pair orbital. Such PeT provides a mechanism for nonradiative deactivation of the excited state (**Figure 2**), leading to a decrease in emission intensity or “quenching” of the fluorescence [5-9].

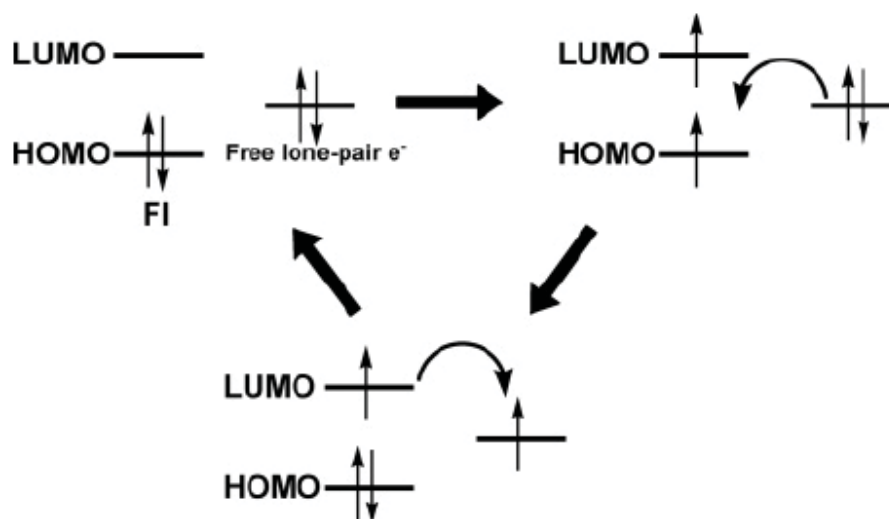


Figure 2 Mechanisms for PeT systems

Photoinduced Charge Transfer (PCT)

Electronic excitation necessarily involves some degree of charge transfer, but in fluorophores containing both electron-withdrawing and electron-donating substituents, this charge transfer may occur over long distances and be associated with major dipole moment changes, making the process particularly sensitive to the microenvironment of the fluorophore. Thus, it can be expected that cations or anions in close interaction with the donor or the acceptor moiety will change the photophysical properties of the fluorophore [10-12]. Upon, for example, cation complexation of an electron donor group within a fluorophore, the electron-donating character of the donor group will be reduced. The resulting reduction of conjugation causes a blue shift of the absorption spectrum together with a decrease of the molar absorptivity. In contrast, metal ion binding to the acceptor group enhances its electron-withdrawing character, and the absorption spectrum is thus red-shifted with an increase in molar absorptivity (**Figure 3**) [13]. The fluorescence spectra should be shifted in the same direction as the absorption spectra, and in addition to these

shifts, changes in the quantum yields and lifetimes can be observed. All these photophysical effects are obviously dependent on the charge and the size of the cation, and therefore, some selectivity is expected [14-24].

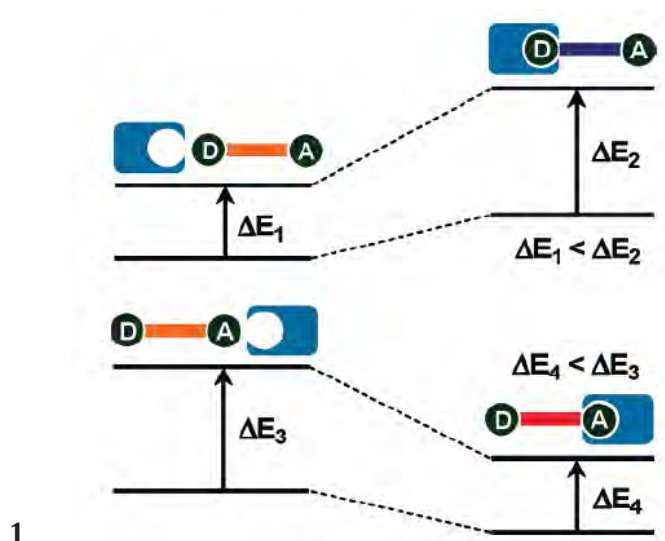


Figure 3 Mechanisms for PCT systems

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Sub-project 1: Highly specific-glucose fluorescence sensing based on boronic anthraquinone derivatives via the GOx enzymatic reaction

This topic was published in *Tetrahedron*, **2012**, 68, 8899-8904 as the attached document #3

1.1 Introduction

Diabetes is a disease, which involves abnormal blood sugar metabolism due to inadequate insulin production. Blood sugar, especially glucose, has regularly been used to diagnose diabetes for over a century. Nowadays, the capability to accurately monitor blood glucose concentrations has proven essentially for diagnosing and curing many metabolic disorders, mainly diabetes.[1] Therefore, reliable and accurate saccharide sensors

are continually developed in order to search for better ones for quantitative analysis and detection of saccharides and their derivatives in physiological media. Moreover, the indispensability of glucose monitoring in the incurable disease is a critical requirement for determination of the glucose levels in nondiabetic acute care patients because high variations in blood glucose level have also been observed in nondiabetic patients as a common sign of acute illness. Patients can modify their lifestyle to better control their diabetes and can use insulin in achieving effective dosing and timing.[2] The important demands for continuous, accurate, and relatively noninvasive glucose sensing methods have motivated methodologies as well as the design of various sensing materials.[3] For specific-glucose sensing, various analytical methods such as amperometry, potentiometry, spectrophotometry, or fluorometry are utilized for the enzymatic measurements of glucose concentration.[4] The traditional commercial glucose test is electrochemical, including a monitor based on the electrical wiring of glucose oxidase, which underlies the direct conversion of the concentration-dependent glucose flux to an electrical current. In this method, the generated hydrogen peroxide is oxidized under a constant working potential, and the extent of oxidation corresponds to the glucose concentration.[5] However, it needs careful calibration because other oxidized products may be easily adsorbed onto the metal electrode surfaces, causing a decrease in activity of the electrode surface and the sensitivity will dramatically decrease.[6] The development of specific sensors for biochemically relevant analytes via chemical reaction has high impact and challenging. Fluorescence sensors with preferential binding to glucose have been developed in the past decade. The obvious advantages of a fluorescence sensor are the extreme sensitivity and lack of damage to the host system. The methodology involves the incorporation of synthetic based receptors, such as boronic acid. Boronic acid based sensors are the best candidate for saccharide probes due to the covalent binding properties of boronic acid with the diol group of sugars in water as widely reported for the last decade.[7] Moreover, most boronic acid derivatives based fluorophores showed the fluorescence emission band at a relatively long wavelength, which is an important role in detecting glucose in blood or serum samples. However, the disadvantages of some boronic acid based fluorescence sensor are the selectivity and a high pKa values, which is not appropriate for physiological pH.[8] Moreover, a number of publications have reported the glucose-specific fluorescence changes with the interference of other saccharides especially fructose and ribose.[9] A few reports exist regarding the selective fluorogenic boronic acid for glucose sensing.[10] As the basic knowledge of boronic acid, aryl boronic acid and boronic ester

react selectively with H_2O_2 to produce a phenolic compound.[11] To overcome the disadvantage of using a boronic-based fluorescence sensor for selective detection of glucose, we took an effort to combine the basic knowledge of the specific-glucose sensing by GOx enzymatic reaction with the new boronic-based anthraquinone for fluorescence changes. Our proposition for the specific glucose fluorescence sensor via the GOx enzymatic reaction is schematically illustrated in Fig. 1.

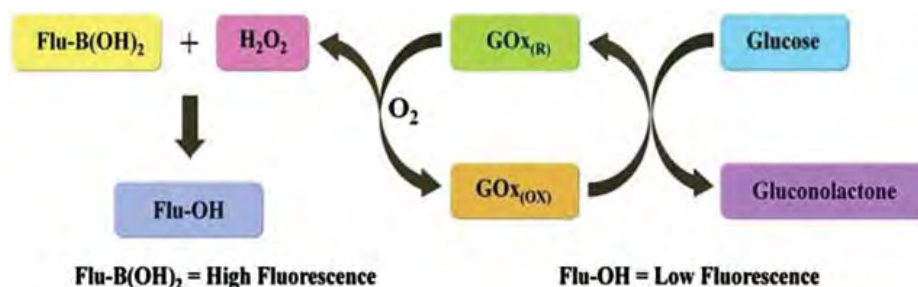
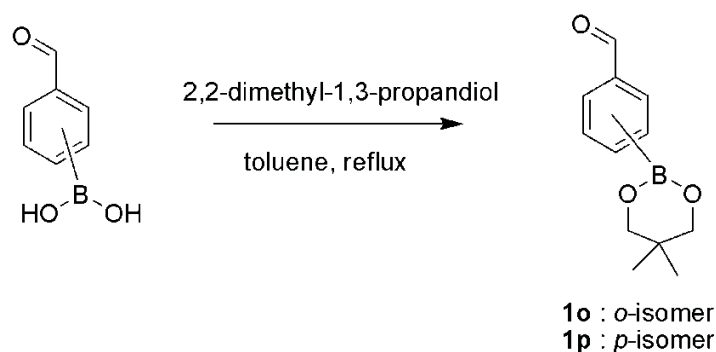


Figure. 1. Schematic illustration of the GOx enzymatic mechanism for glucose detection using boronic-based fluorescence sensors.

For our hypothesis, the H_2O_2 generated by the reaction between glucose and glucose oxidase would convert the boronic acid to the phenol based fluorescence sensor and the fluorescence change would be related to the concentration of glucose. Although, most organic fluorophores can exhibit fluorescence quenching by H_2O_2 , we designed the organic fluorophore containing boronic acid for specific detection of glucose upon the GOx enzymatic reaction without the interference of glucose binding to the boronic acid directly.

1.2 Experimental section

- Preparation of R-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl)benzaldehyde (R = 2 : 1o, 4 : 1p).



Into a two-neck round bottom flask equipped with a magnetic bar and a Dean-Stark equipment to remove water, the corresponding formyl phenyl boronic acid (0.150 g, 1 mmol) and 2,2-dimethyl-1,3-propanediol (0.104 g, 1mmol) were heated at reflux in toluene

(30 mL) for 12 hours. Subsequently, the solvent was evaporated under reduced pressure to give a crude product as clear oil for ortho isomer (**1o**) and as a white solid for para isomer (**1p**). The crude product was used in the next step without purification.

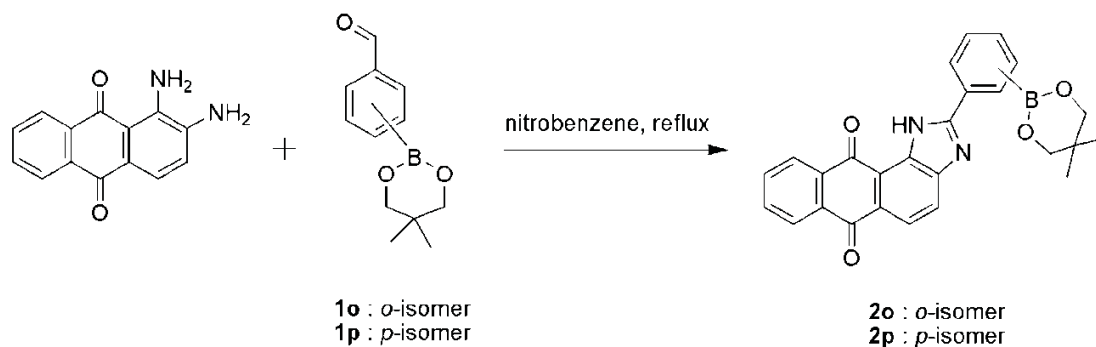
Characterization data for **1o** and **1p**

¹H NMR (DMSO-*d*₆, 400 MHz)

Compound 1o : [ortho isomer] δ (ppm) : 10.52 (s, 1H), 7.89 (t, 1H, *J* = 7.6 Hz), 7.79 (d, 1H, *J* = 7.2 Hz), 7.24 (t, 1H, *J* = 7.6 Hz), 7.16 (d, 1H, *J* = 7.2 Hz), 3.81 (s, 2H), 3.48 (s, 2H), 1.07 (s, 3H), 0.90 (s, 3H).

Compound 1p : [para isomer] δ (ppm) : 10.03 (s, 1H), 7.95 (d, 2H, *J* = 8.0 Hz), 7.85 (d, 2H, *J* = 8.0 Hz), 3.79 (s, 4H), 1.03 (s, 6H).

- Preparation of 2-(R-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl) phenyl)-1H-anthra[1,2-d]imidazole-6,11-dione (R = 2 : **2o**, 4 : **2p**).



Into a two-neck round bottom flask equipped with a magnetic bar, the corresponding formylphenylboronate ester (0.218 g, 1 mmol) in nitrobenzene (25 mL) was added drop wise to a solution of 1,2-diamino-1,4-anthraquinone (0.238 g, 1 mmol) in nitrobenzene (75 mL). The reaction mixture was slowly heated up to 150 °C for 1 day under nitrogen atmosphere. The solution was cooled to room temperature and then the precipitate slowly formed. The precipitate was filtered and washed with diethyl ether to give a brown solid the protecting product **2o** (0.196 g, 45 %yield) and a yellow solid of the protecting product **2p** (0.262 g, 60 %yield).

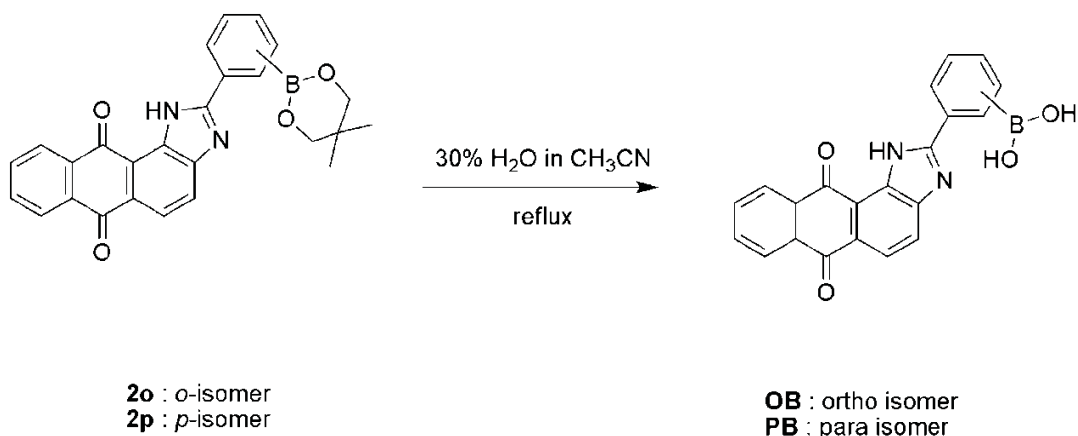
Characterization data for 2o and 2p

¹H NMR (DMSO-*d*₆, 400 MHz)

Compound 2o :[ortho isomer] δ (ppm) : 13.56 (s, 1H), 8.41 (d, 1H, *J* = 6.4 Hz), 8.25-8.13 (m, 4H), 7.94-7.92 (m, 2H), 7.57-7.50 (m, 3H), 3.70 (s, 4H), 1.07 (s, 6H).

Compound 2p :[para isomer] δ (ppm) : 10.18 (s, 1H), 8.40 (t, 1H, *J* = 8.2 Hz), 8.29-8.21 (m, 4H), 8.13-8.05 (m, 2H), 7.97-7.93 (m, 3H), 3.78 (s, 2H), 3.11 (s, 2H), 0.97 (s, 3H) 0.72 (s,3H)

- **Preparation of R-(6,11-dioxo-6,11-dihydro-1H-anthra[1,2-d]imidazol-2-yl)phenylboronic acid (R = 2 : OB, 4 : PB).**



Protecting groups of 2-(R-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl) phenyl)-1H-anthra[1,2-d]imidazole-6,11-dione (R = 2 : **2o**, 4 : **2p**) (0.436 g, 1 mmol) were removed by refluxing in 30 mL of 30% H₂O:CH₃CN. The yellow solid of product **OB** and brown solid of product **PB** was filtered and washed with diethyl ether to provide a desired product in a quantitative yield.

Characterization data for OB and PB

¹H NMR (DMSO-*d*₆, 400 MHz)

Compound OB : [ortho isomer] δ (ppm) : 13.76 (s, 1H), 8.26-8.21(m, 2H), 8.14 (d, 2H, J = 6.8 Hz), 7.99 (d, 2H, J = 6.0 Hz), 7.94-7.92 (m, 1H), 7.75 (d, 1H, J = 8.4 Hz), 7.68 (d, 2H, J = 6.8 Hz).

Compound PB : [para isomer] δ (ppm) : 10.97 (s, 1H), 9.21 (d, 1H, J = 7.6 Hz), 9.11-9.00 (m, 3H), 8.96-8.86 (m, 2H), 8.78-8.74 (m, 3H), 7.73 (d, 1H, J = 8.4 Hz).

^{13}C NMR (DMSO- d_6 , 100.6 MHz)

Compound OB : [ortho isomer] δ (ppm) : 182.36, 182.00, 181.50, 159.60, 148.58, 133.60, 132.59, 132.33, 131.91, 129.68, 129.23, 128.49, 128.04, 126.84, 126.66, 126.23, 126.12, 124.74, 121.60, 120.93, 118.58.

Compound PB : [para isomer] δ (ppm) : 183.02, 182.25, 160.21, 149.19, 137.13, 132.99, 132.88, 130.07, 130.01, 128.00, 127.31, 126.99, 126.69, 126.13, 124.90, 124.12, 120.97, 120.91, 119.67, 118.57, 115.54.

High Resolution Mass spectrometer (ESI-HRMS)

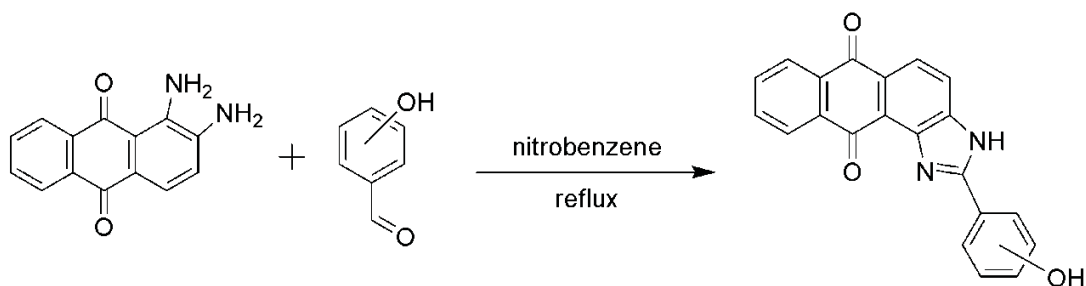
Compound OB : m/z : for ($M + \text{CH}_3\text{OH} + \text{H}_2\text{O} + \text{H}^+$) Anal. = 419.123

Calc. = 419.13

Compound PB : m/z : for ($M + \text{CH}_3\text{OH} + \text{H}_2\text{O} + \text{H}^+$) Anal. = 419.097 (**Figure A.24**)

Calc. = 419.13

- Preparation of 2-(*R*-hydroxyphenyl)-3H-anthra[2,1-*d*]imidazole-6,11-dione (**R** = 2 : **OO**, 4 : **PO**).



OO : ortho isomer
PO : para isomer

Into a two-neck round bottom flask equipped with a magnetic bar, the corresponding hydroxybenzaldehyde (0.122 g, 1 mmol) in nitrobenzene (10 mL) was added drop wise to a solution of 1,2-diamino-1,4-anthraquinone (0.238 g, 1 mmol) in nitrobenzene (40 mL). The reaction mixture was slowly heated up to 150 °C for 48 hours under nitrogen atmosphere. The solution was cooled to room temperature and then, the precipitate slowly formed. The precipitate was filtered and washed with diethyl ether (20 ml) to give a dark brown solid of the product **OO** (0.384 g, 64 %yield) and give a brown solid of the product **PO** (0.265 g, 78 %yield).

Characterization data for OO and PO

¹H NMR (DMSO-*d*₆, 400 MHz)

Compound OO δ (ppm) : 12.73 (s, 1H), 12.01 (s, 1H), 8.42 (d, 1H, *J* = 8.0 Hz), 8.16 (s, 2H), 8.07 (m, 1H), 8.71 (d, 1H, *J* = 16 Hz), 7.90 (s, 2H), 7.42 (t, 1H, *J* = 7.6 Hz), 7.09 (d, 1H, *J* = 8.4 Hz), 7.03 (t, 1H, *J* = 7.2 Hz).

Compound PO δ (ppm) : 12.86 (s, 1H), 10.18 (s, 1H), 8.26 (d, 2H, *J* = 7.6 Hz), 8.17 (s, 2H), 8.00 (s, 2H), 7.89-7.88 (m, 2H), 6.91 (d, 2H, *J* = 8 Hz).

- The enzymatic probe studies of the boronic anthraquinone based sensors

- The photochemical studies of boronic anthraquinone based sensors and their derivatives for enzymatic probe system, OB, PB, OO and PO for glucose oxidase

UV/Vis spectroscopic screening test of sensors

The sensors were prepared in spectroscopic grade DMSO at concentrations of 2.00×10^{-4} mol/L as a stock solution. The solution of sensors were mixed with 0.1 M HEPES buffer pH 7.4 to give a final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution. 2.00 mL of sensor solution was placed in a 100.0 mm width quartz cell. UV/Vis spectra were recorded at 25°C after each addition.

Fluorescence spectroscopic screening test of sensors

Typically, the sensors were prepared in spectroscopic grade DMSO at concentrations of 2.00×10^{-4} mol/L as a stock solution. The solution of sensors were mixed with 0.1M HEPES buffer pH 7.4 to give a final concentration of sensors at 2.00×10^{-5} mol/L, 10% DMSO in aqueous solution. 2.00 mL of sensor solution was placed in a 100.0 mm width quartz cell. Fluorescence spectra were recorded at 25°C after each addition.

- The complexation studies of the boronic anthraquinone based sensors with glucose using fluorescence titration experiments

Typically, the sensors were prepared in spectroscopic grade DMSO at concentrations of 2.00×10^{-4} mol/L as a stock solution. The stock solutions of glucose were prepared at concentrations of 6.00×10^{-3} mol/L in 0.1 M HEPES buffer pH 7.4. In a 5.0 mL beaker, 0.30 mL of the stock solution of sensor was mixed with the portions of stock solution of glucose to give final concentration of glucose according to **Table 3.1**. After volume were adjusted to 3.00 mL with 0.1M HEPES buffer pH 7.4 to give a final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution, the mixtures was stirred at room temperature for 10 minutes and placed in a 100.0 mm width quartz cell and then fluorescence spectra were recorded at 25°C.

- The reaction studies of the boronic anthraquinone based sensors with hydrogen peroxide using fluorescence titration experiments

Typically, the sensors were prepared in spectroscopic grade DMSO at concentrations of 2.00×10^{-4} mol/L as a stock solution. The stock solutions of hydrogen peroxide were prepared at the concentrations of 6.00×10^{-3} mol/L in 0.1M HEPES buffer pH 7.4. In a 5.0 mL vial, 0.30 mL of the stock solution of sensor was mixed with the portions of stock solution hydrogen peroxide to give final concentration of hydrogen peroxide according to **Table 3.2**. After volume were adjusted to 3.00 mL with 0.1M HEPES buffer pH 7.4 to give a final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution. The mixtures was stirred at room temperature for 60 minutes and placed in a 100.0 mm width quartz cell and then fluorescence spectra were recorded at 25°C.

- Fluorescence measurement for the optimizing condition of the enzymatic probe system

Fluorescence measurements in this section were performed under the condition following the mentioned above.

- The studies on the effect of unit of glucose oxidase

In a 10.0 mL vial, 0.30 mL of 2.00×10^{-4} mol/L of a sensor in spectroscopic DMSO was mixed with 100 μ L of 6.00×10^{-2} mol/L glucose in 0.1M HEPES buffer pH 7.4 and adjusted to 3.00 mL to give final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution. Then glucose oxidase (GOx) in various portions. was added to the solution mixture of the sensor and the glucose. After stirred for 60 minutes, the mixture sample was placed in a 100.0 mm width quartz cell, and the fluorescence spectra were then recorded at room temperature.

- The studies on the effect of temperature

In a 10.0 mL vial, 0.30 mL of 2.00×10^{-4} mol/L of a sensor in spectroscopic DMSO was mixed with 100 μ L of 6.00×10^{-2} mol/L glucose in 0.1M HEPES buffer pH 7.4 and adjusted to 3.00 mL to give final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution. Then, 6 units of glucose oxidase were added to the solution mixture of the sensor and the glucose. One mixture was stirred at room temperature and another was stirred at 37°C for 60 minutes and placed in a 100.0 mm width quartz cell and then, the fluorescence spectra were recorded at room temperature.

- The studies on the reaction time

In a 100.0 mm width quartz cell, 0.30 mL of 2.00×10^{-4} mol/L of a sensor in spectroscopic DMSO was mixed with 100 μ L of 6.00×10^{-2} mol/L glucose in 0.1M HEPES buffer pH 7.4 and adjusted to 3.00 mL to give final concentration of sensors at 2×10^{-5} mol/L in 10% DMSO aqueous solution. Then 6 units of glucose oxidase were added to the solution mixture of the sensor and the glucose. The mixture was stirred at room temperature and then, the fluorescence spectra were recorded at room temperature every 5 minutes.

- Fluorescence measurement of boronic anthraquinone based sensors (OB) in optimum condition of the enzymatic probe system for glucose detected by glucose oxidase

Typically, the sensors were prepared in spectroscopic grade DMSO at concentrations of 2.00×10^{-4} mol/L as a stock solution. The stock solutions of glucose were prepared at concentrations of 6.00×10^{-3} mol/L in 0.1M HEPES buffer pH 7.4. In a 10.0 mL vial, 0.30 mL of the stock solution of sensor was mixed with the portions of stock solution of glucose to give final concentration of glucose. After volume were adjusted to 3.00 mL with 0.1M HEPES buffer pH 7.4 to give a final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution, then 6 units of glucose oxidase were added to the solution mixture of the sensor and the glucose. The mixtures was stirred at 37°C for 60 minutes and placed in a 100.0 mm width quartz cell and then, the fluorescence spectra were recorded at room temperature

- The studies of limit of detection of enzymatic probe for glucose

The sensors were prepared in spectroscopic grade DMSO at concentrations of 2.00×10^{-4} mol/L as a stock solution. The solution of sensors were mixed with 0.1M HEPES buffer pH 7.4 to give a final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution. 2.00 mL of sensor solution was placed in a 100.0 mm width quartz cell. Fluorescence spectra were recorded at room temperature for 10 times.

- Fluorescence measurement of boronic anthraquinone based sensors (OB) in optimum condition of the enzymatic probe system for glucose detection and other competitive saccharides by glucose oxidase

In a 10.0 mL vial, 0.30 mL of 2.00×10^{-4} mol/L of a sensor in spectroscopic DMSO was mixed with 100 μ L of 6.00×10^{-2} mol/L of stock solution including D-glucose, D-galactose, D-fructose, D-sucrose and D-maltose in 0.1M HEPES buffer pH 7.4 and all solutions was adjusted to 3.00 mL to give final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution. Then 6 units of glucose oxidase were added to the solution mixture of the sensor and the sugar. The mixture solution was stirred at 37°C for

60 minutes and placed in a 100.0 mm width quartz cell and then fluorescence spectra were recorded at room temperature.

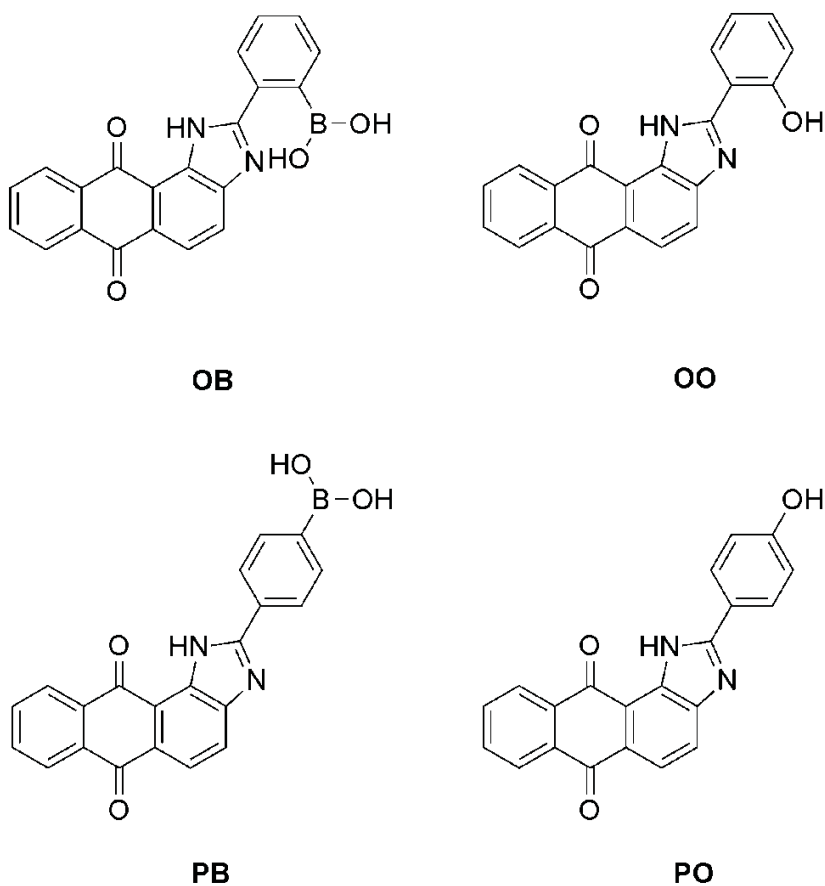
1.3 Results and discussion

Design concept of enzymatically activated sensors for glucose oxidase

Our research has focused on the design and synthesis of the molecule which can be enzymatic probes for biomolecule such as sugar, protein, etc in vivo. These make use of biocomponents capable of recognizing bio- or chemical species and transforming them into a product through a chemical reaction. This type of biosensor is represented mostly by enzymatic biosensors, which make use of specific enzymes or their combinations. Typically, the fluorogenic sensors consist of receptor units that can react with target biomolecule and signaling units connecting with the spacer. In the field of supramolecule chemistry, recognition events occur via non-covalent interactions including hydrogen bonding, electrostatic interactions, ion-dipole interactions, cation- π interactions and π - π stacking interactions. It is well known that those interactions played an important role in biological systems due to the selectivity and sensitivity. In natural system, an effective non-covalent recognition of host and guest are derived from complicated molecules or systems that are able to expel or strip the solvated water molecules from the binding site of the host guest molecule resulting in perfect recognitions. However, the vivo system has aqueous everywhere then many synthetic receptors based on non-recognition are still incapable function in aqueous system because guest molecules are solvated by water. Consequently, the synthetic receptors used in water are less effective. Moreover, the vivo system has many molecules that able interfere the interaction between the synthetic receptors and the target molecules. In order to overcome the solvation problem and interference from other molecules, the enzymatically activated system was introduced to case in form of sensory molecule. In our research, the boronic acid was candidate for enzymatically activated system by glucose oxidase for glucose detection. Typically, the glucose oxidase produces the gluconic acid and the generated hydrogen peroxide. The previous works reported [12] that boronic acid can be oxidized to be hydroxyl group by hydrogen peroxide. This approach motivates to design the boronic based receptor to detect glucose by enzymatic probe using fluorescence spectroscopy.

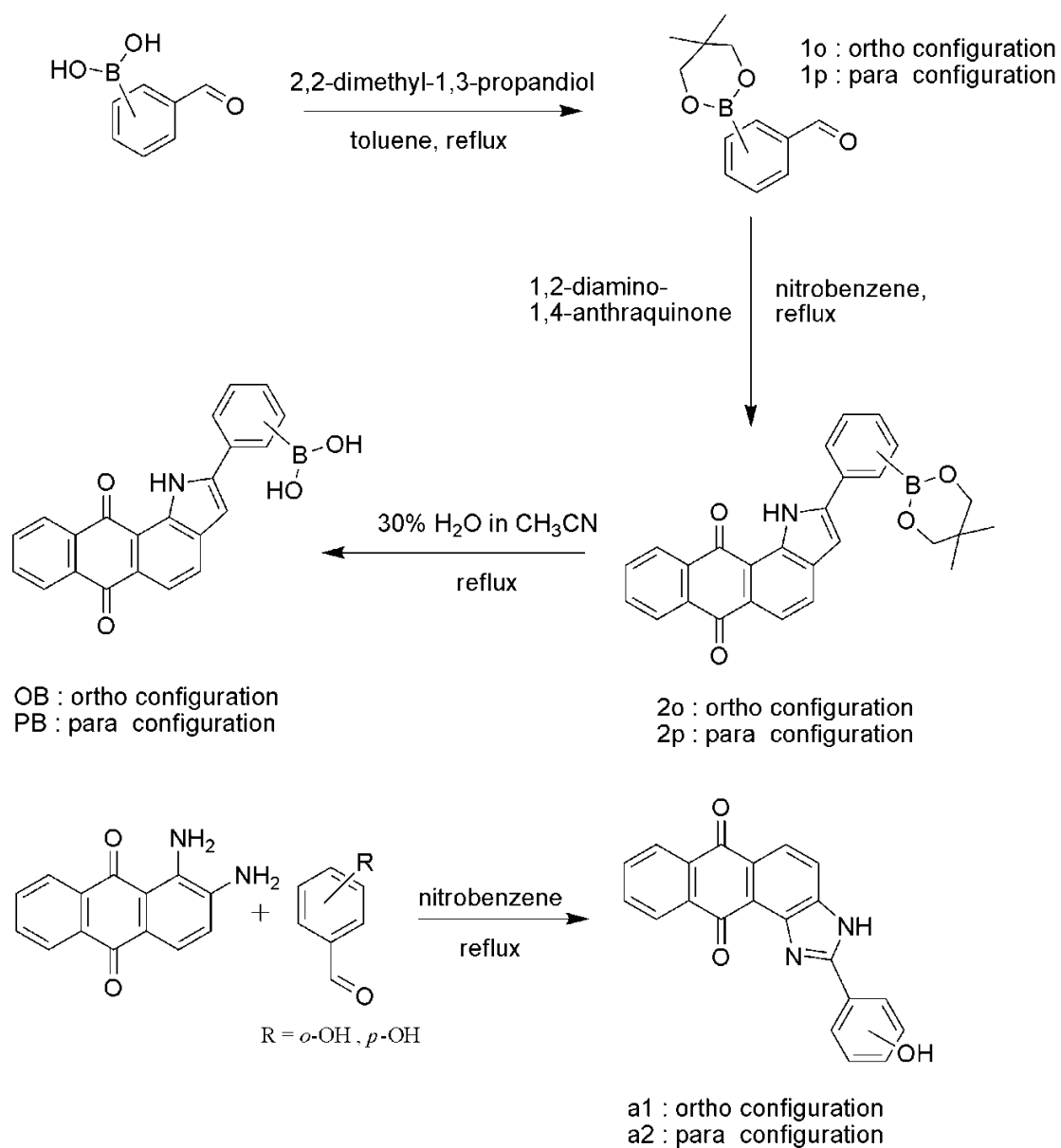
There are two main types of optical biosensors: The first exploits any changes that can occur in the intrinsic optical property of the biomolecule as a result of its interaction

with the target analyte. Such changes can occur in absorbance, emission, polarization, or luminescence decay time of a receptor. Such sensors are not numerous because their sensitivity is usually low, and because many effects occur in the deep UV where spectroscopy has academic merits but is difficult to implement when it comes to analyzing complex (such as environmental or clinical) samples. An additional challenge when using intrinsic biosensors consists of the separation of the shortwave signal from background fluorescence (or absorbance). Enzymes using FAD as a coenzyme are examples of more longwave absorbing receptors that undergo intrinsic spectral changes on binding a ligand (during catalytic conversion), as are some cytochromes and hemoglobin. The second type of biosensor is making use of optical labels and probes of various kinds. This requires the biomolecule to be covalently labeled (an extra step) but enables the analytical wavelength(s) to be shifted to almost any desired value. Moreover, luminescence decay times and anisotropy can be adjusted to the desired values, and effects such as dynamic or static quenching can be exploited in a more systematic manner. Not surprisingly, luminescent labels are widely used for this purpose. In recent years, the use of luminescent nanoparticles has strongly increased. Regarding to signaling system, optical output such as color, absorption spectra and especially fluorescence spectra was usually due to sensitivity and uncomplicated instrumental readout. Hypothetically, the conjugation of an electron acceptor such a quinine derivative with electron donor such an imidazole as a main acceptor-donor segment (main A-D system) performs intramolecular charge transfer signaling system which is sensitive to a small perturbation. Conjugative connecting of reactive recognition site as a boronic acid to main A-D site was expected to afford the large response to the recognition events. Designed receptors based on boronic anthraquinone and derivatives were depicted in **Scheme 1**



Scheme 1 Structures of designed receptors based on anthraquinone, imidazole and boronic acid

From these mentioned above, anthraquinone was chosen to fabricate a main electron acceptor segment of the synthesized receptors and to study the enzymatic probe for glucose by glucose oxidase catalytic enzyme measured by fluorescent measurements. The synthetic pathway is shown in **Scheme 2**.

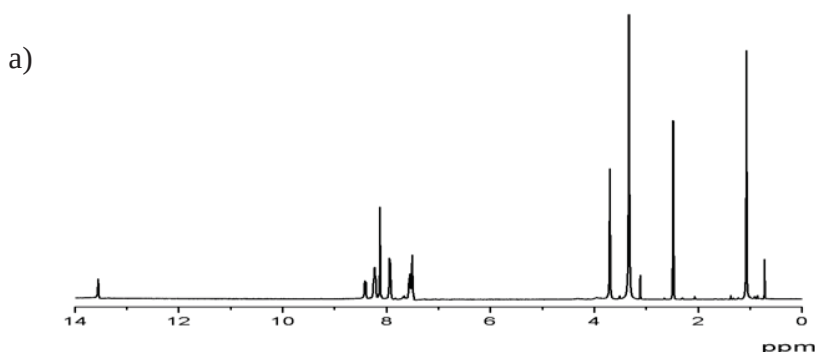


Scheme 2 Synthesis pathway of boronic anthraquinone (**OB** and **PB**) and their derivatives (**OO** and **PO**)

Synthesis and characterization of boronic anthraquinone based sensors and their derivatives

In order to avoid the side reaction due to weak Lewis acid properties of boronic acid, the synthetic pathway was started by protecting the boronic group of appropriated formyl phenyl boronic acids using 2,2-dimethyl-1,3-propanediol in refluxing toluene. Dean-stark apparatus was equipped with the reaction flask in order to remove water from the condensation reaction. After refluxing overnight and removing of toluene, the clear oil of the protected formyl phenyl boronate products were obtained. The crude product was used in the next step without the purification and confirmed by ^1H -NMR spectroscopy by the appearance of ethylene chain as a singlet peak at 3.81 and 3.48 ppm for ortho isomer (**1o**) and 3.79 ppm for para isomer (**1p**).

The key step of the synthesis is the oxidative condensation of diamine and aldehyde to yield the desired heterocyclic imidazole. Protecting of compounds **OB** and **PB** (**2o** and **2p**) were prepared by condensation of 1,2-diamino-1,4-anthraquinone with the corresponding formyl phenyl boronate ester in refluxing nitrobenzene and the reaction was kept at 150 °C for 1 day. Compounds **2o** and **2p** were separated from the reaction mixture by precipitation to obtain a brown solid of the protecting product **2o** (45 %yield) and a yellow solid of the protecting product **2p** (60 %yield). Structure of the heterocyclic products **2o** and **2p** were assured by ^1H -NMR spectroscopy (**Figure 1**). Interestingly, NH imidazole protons were found at 13.56 and 10.18 ppm for **2o** and **2p**, respectively. This is indicative of a very high acidic proton due to the intramolecular hydrogen bonding between NH-imidazole and oxygen of anthraquinone.



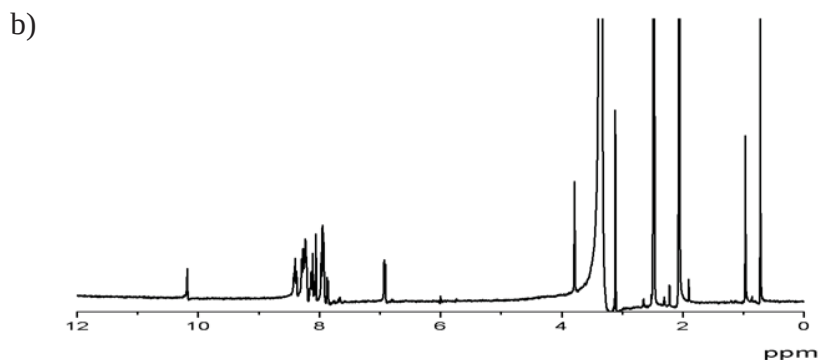


Figure 1 ^1H -NMR spectra (400 MHz, $\text{DMSO-}d_6$) for a) **2o** and b) **2p**

In order to remove the protecting boronate group, compounds **2o** and **2p** were refluxed in 30% H_2O in CH_3CN to give the protonated sensors **OB** as yellow solids and brown solids of product **PB**. Compounds **OB** and **PB** were characterized by ^1H -NMR, MADI-TOF and High resolution mass spectroscopy. As displayed in **Figure 2**, ^1H -NMR spectra showed that the protecting groups were completely removed by the appearance of hydroxyl signals of the boronic acid moiety and the vanishing of proton signals of the ethoxy chains. Considering each isomer, the ^1H -NMR spectrum of **OB** clearly showed the feature of the desired compound by the appearance of signal of acidic protons of imidazole signal at 13.76 ppm.

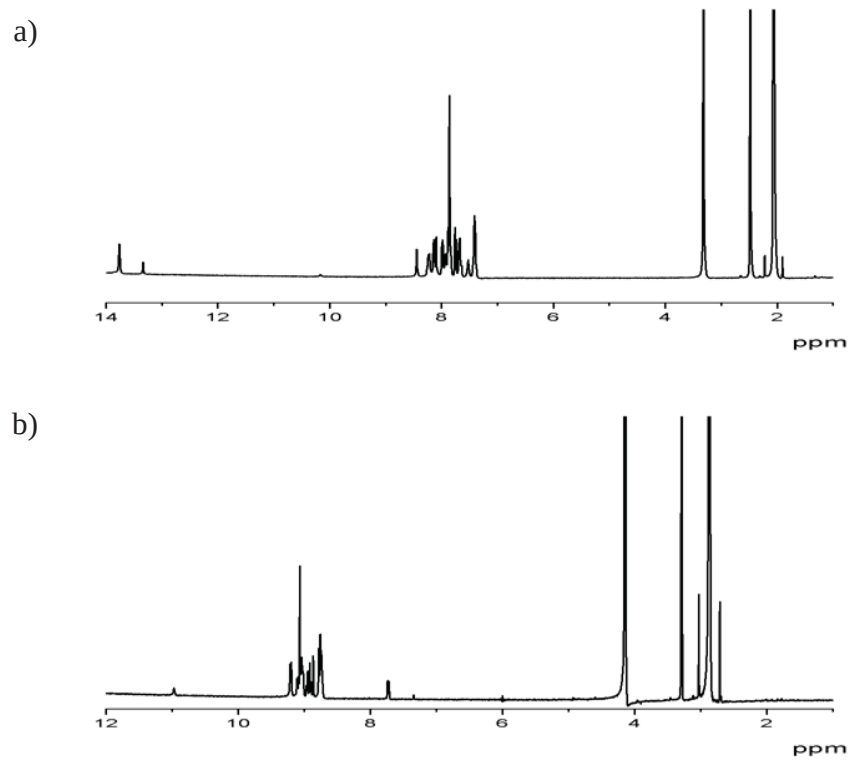


Figure 2 ^1H -NMR spectra (400 MHz, $\text{DMSO-}d_6$) for a) **OB** and b) **PB**

The enzymatic probe studies of the boronic anthraquinone based sensors

-The photochemical studies of boronic anthraquinone based sensors and their derivatives for enzymatic probe system of OB, PB, OO and PO for glucose oxidase

The absorption properties of boronic anthraquinone (**OB** and **PB**) based sensors and their derivatives (**OO** and **PO**) showed a light yellow color solution and the visible absorption bands at 415, 398, 408 and 424 nm for **OB**, **PB**, **OO** and **PO**, respectively (shown in **Table 4.1** and **Figure 3**). From these results, the light yellow color and the visible absorption bands obtained the effect from the migration of electron density from electron donating group of amino on imidazole ring to electron acceptor group of anthraquinone. Compared to the parent compound, 1,2-diamino-1,4-anthraquinone ($\lambda_{\text{max}} = 495$ nm, $E_g \approx 2.50$ eV), boronic anthraquinone based sensors provided a large energy gap ($E_g \approx 2.98$ -3.12 eV) due to the attachment of electron deficient group (boronic acid) to the structure.

Table 1 Photophysical properties of the boronic anthraquinone (**OB** and **PB**) based sensors and their derivatives (**OO** and **PO**) in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

Receptors	λ_{abs} (nm)	λ_{em} (nm)	Φ_f^a
OB	415	609	0.322
PB	398	589	0.231
OO	408	n.d. ^b	0.015
PO	424	n.d. ^b	0.031

^a Quantum yields were determined using quinine sulfate as the standard (Φ_{STD}) 0.508 in 1N H₂SO₄.

^b Values cannot be determined.

As expected, the boronic anthraquinone based sensors exhibit emission properties as listed in **Table 1**. Their quantum yield in 10% DMSO with 0.1M HEPES buffer at pH 7.4, in aqueous solution was quite low in the attribution of the characteristic of low lying n-

π^* excited state in anthraquinone compound resulting in dominating of intersystem crossing relaxation pathway [121].

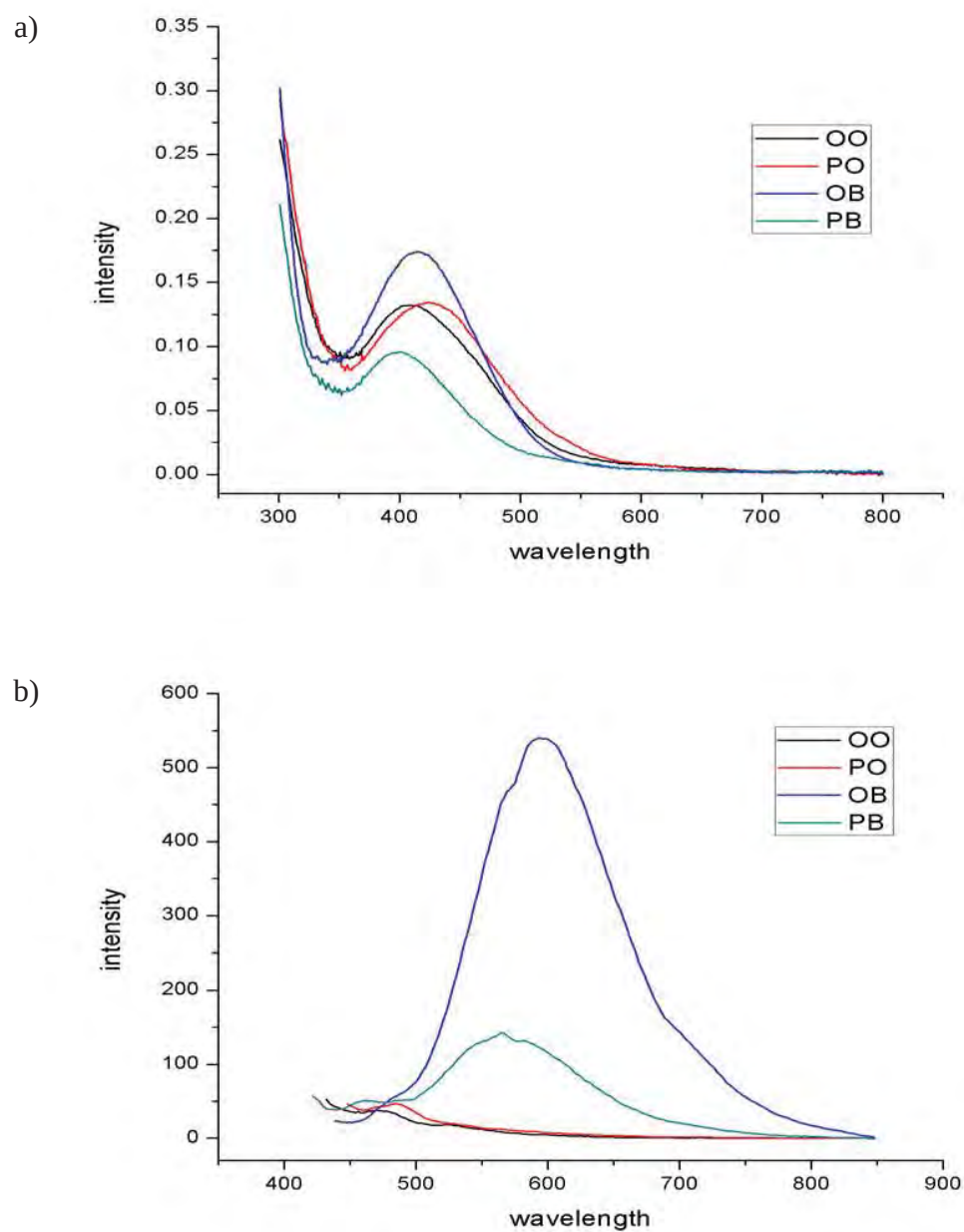


Figure 3 a) Absorption spectra and **b)** emission spectra of **OO**, **PO**, **OB** and **PB** in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

- The complexation studies of the boronic anthraquinone based sensors with glucose using fluorescence titration experiments

The aim of this work focused on the evaluation of the boronic anthraquinone based sensors used for detection of glucose on enzymatically activated system. A glucose oxidase is an enzyme that can oxidize the glucose to produce hydrogen peroxide and gluconic acid. To apply **OB** and **PB** in complicated systems, they were expected to detect the hydrogen peroxide that was generated from the oxidation of glucose by glucose oxidase which is selective enzyme for only glucose. Our hypothesis is that the amount of the generated hydrogen peroxide was proportional to the amount of glucose. Since **OB** and **PB** contain boronic group which can actually bind with saccharides. The complexation abilities of sensors (**OB** and **PB**) with various concentration of glucose were investigated by the fluorometric titration in 10% DMSO with 0.1M HEPES buffer at pH 7.4. The fluorescence response of **OB** showed the insignificant changes upon adding glucose (shown in **Figure 4**) while the fluorescence response of **PB** showed the large spectral change upon adding glucose (shown in **Figure 5**). At high equivalent of glucose, the solution of **PB** became less emissive with a 2-fold fluorescence compared to the absence of glucose. Considering the titration curve (**Figure 6**) of **OB** and **PB** with glucose, without glucose oxidase in system, the results showed that **PB** can interact directly with glucose while no effect of glucose towards **OB** was observed.

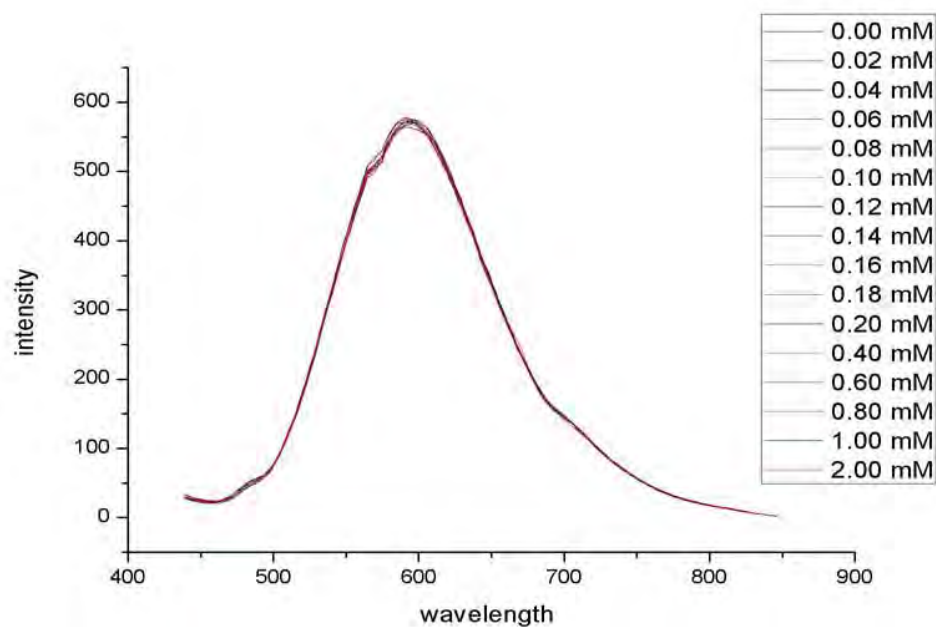


Figure 4 Emission spectra of 20 μM **OB** titrated with glucose in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

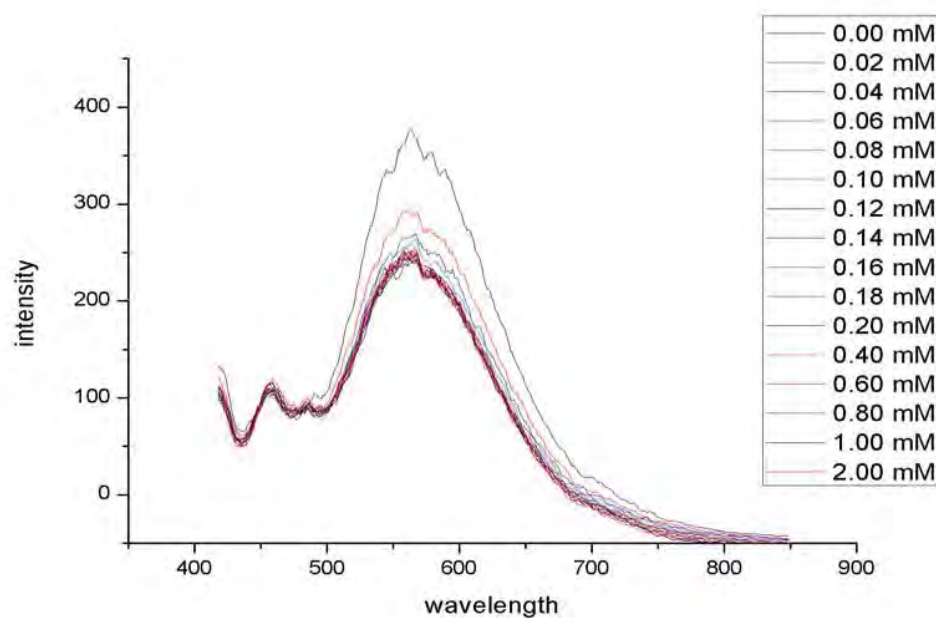


Figure 5 Emission spectra of 20 μM **PB** titrated with glucose in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

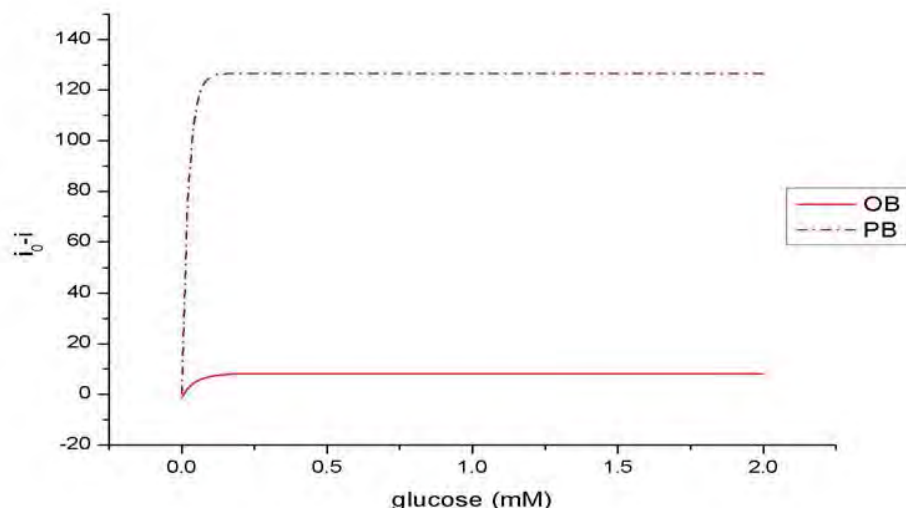


Figure 6 Emission intensity at 609 and 589 nm of 20 μ M **OB** and **PB**, respectively and titrated with glucose in 10% DMSO with 0.1M HEPES buffer at pH 7.4. i_0 is the intensity in the absence of glucose. i is a variation of the emission intensity in the presence of glucose.

- The reaction studies of the boronic anthraquinone based sensors with hydrogen peroxide using fluorescence titration experiments

Next, we would like to check the direct reaction of boronic acid and hydrogen peroxide by fluorescence titration in 10% DMSO with 0.1M HEPES buffer at pH 7.4. From the fluorescence spectra and titration curve of **OB** and hydrogen peroxide (**Figure 7** and **Figure 9**), they exhibited the fluorescence quenching at 609 nm which is proportional to the concentration of added hydrogen peroxide added and is constant at the high concentration of H_2O_2 . In the case of **PB** and H_2O_2 the fluorescence spectra and titration curve (**Figure 8** and **Figure 9**) at 589 nm increased after adding a small amount of H_2O_2 and then were quench and gradually constant upon the addition of more amount of H_2O_2 . These results suggested that **OB** should be a suitable sensory molecule for detection of H_2O_2 and future applied to evaluate the glucose in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

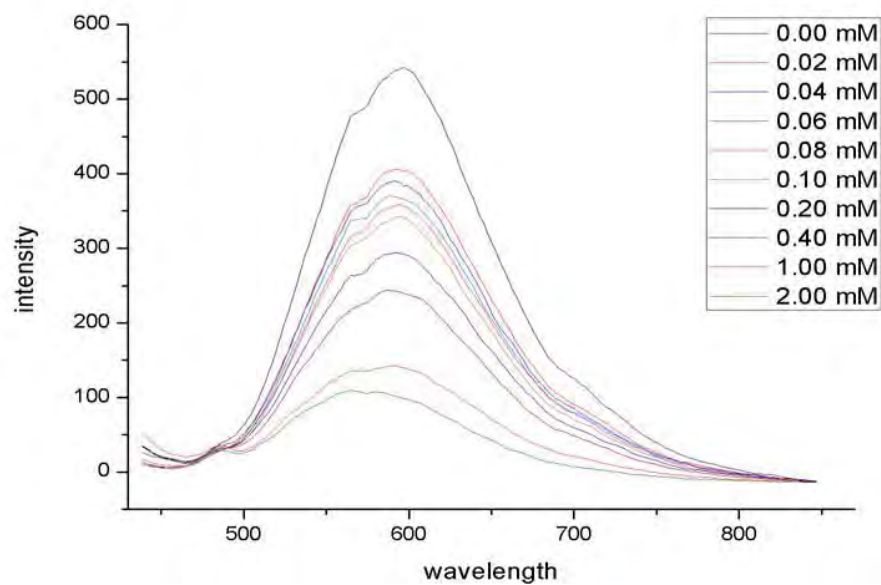


Figure 7 Emission spectra of 20 μM **OB** titrated with hydrogen peroxide in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

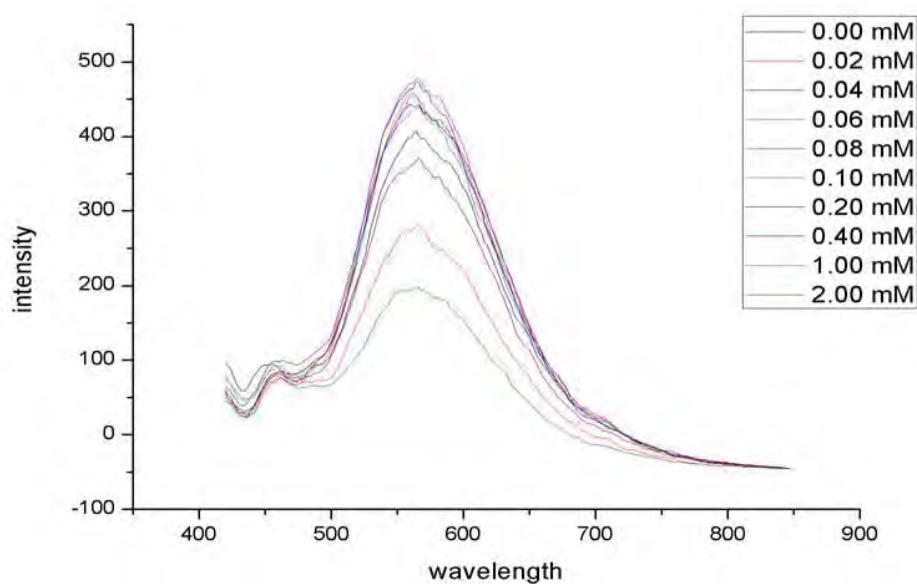


Figure 8 Emission spectra of 20 μM **PB** titrated with hydrogen peroxide in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

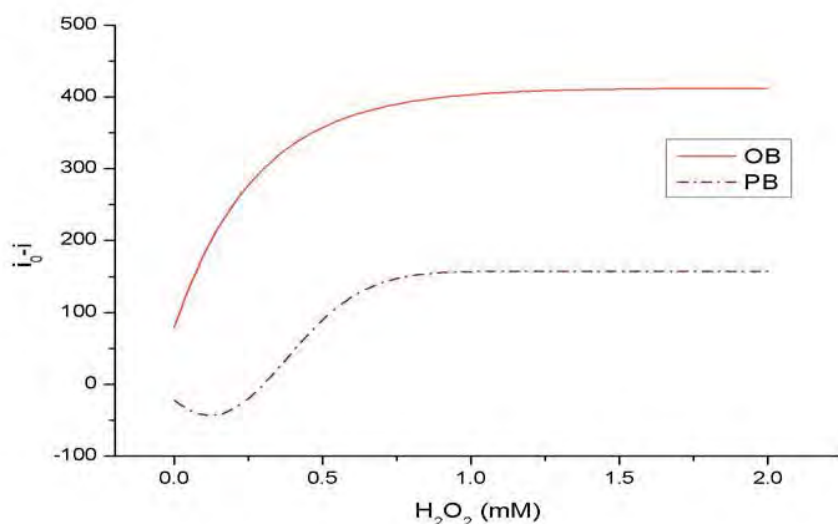
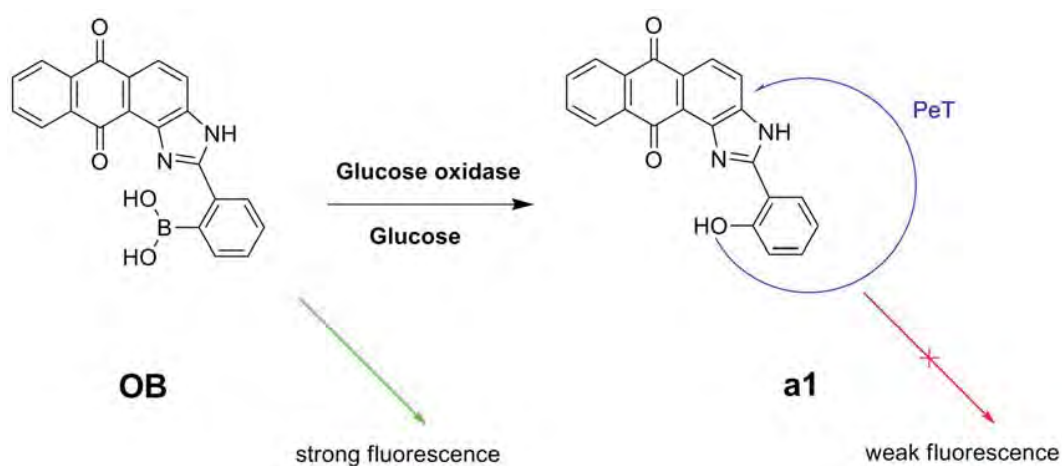


Figure 9 Emission intensity at 609 and 589 nm of 20 μ M **OB** and **PB**, respectively and titrated with hydrogen peroxide in 10% DMSO with 0.1M HEPES buffer at pH 7.4. i_0 is the intensity in the absence of hydrogen peroxide. i is a variation of the emission intensity in the presence of hydrogen peroxide.

- Fluorescence measurement for the optimizing condition of the enzymatic probe system

As described above, **OB** is a good candidate for fluorogenic sensor to detect glucose by enzymatic probe with glucose oxidase. The assumed mechanism for detection of glucose was illustrated in **Scheme 3** which described that the characteristic **OB** exhibited a strong fluorescence in the absence of glucose in enzymatic probe with glucose oxidase. After adding glucose in the solution of **OB** and glucose oxidase, the boronic acid was oxidized by H_2O_2 generated from the reaction of glucose and glucose oxidase providing hydroxyl group of **OB** resulting in a weak fluorescence intensity. Therefore, we must optimize the condition for enzymatic probe system.



Scheme 3 Reaction scheme of **OB** before and after the reaction with glucose oxidase

- The studies on the effect of amount of glucose oxidase

To optimize the appropriate amount of glucose oxidase for the high efficiency of enzymatic probe system by fluorescence measurements. The amount of unit of glucose oxidase was used in this enzymatic probe for **OB** examined from 0 to 9 units of glucose oxidase. As the results shown in **Figure 10**, the fluorescence response of **OB** at 6 and 9 units of glucose oxidase displayed a large fluorescence quenching of **OB**. It indicated that this amount of glucose oxidase could generate enough H_2O_2 to interact with boronic acid based sensor (**OB**). However, we have utilized 6 units of glucose oxidase in optimal condition for enzymatic probe system.

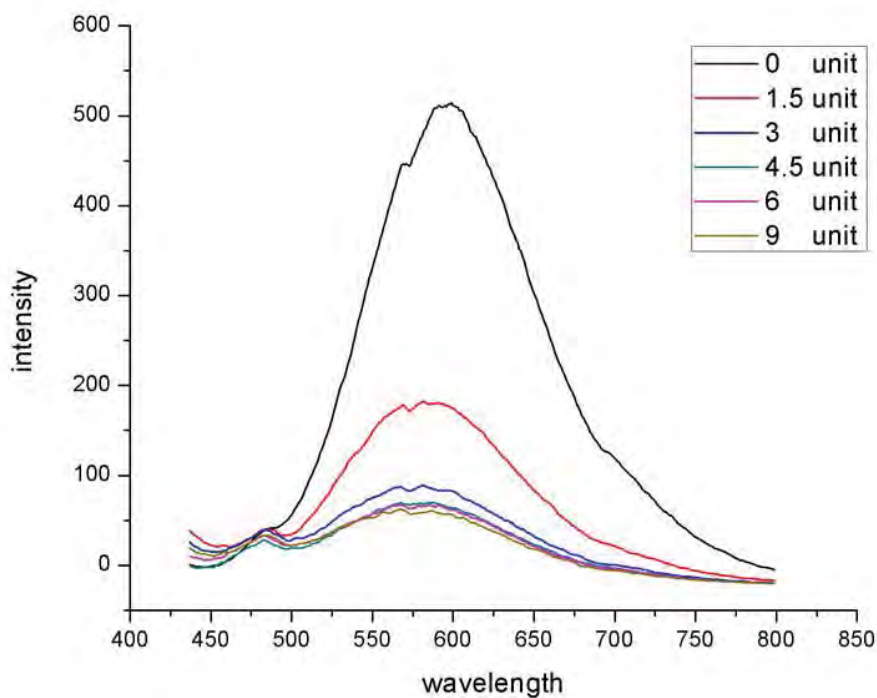
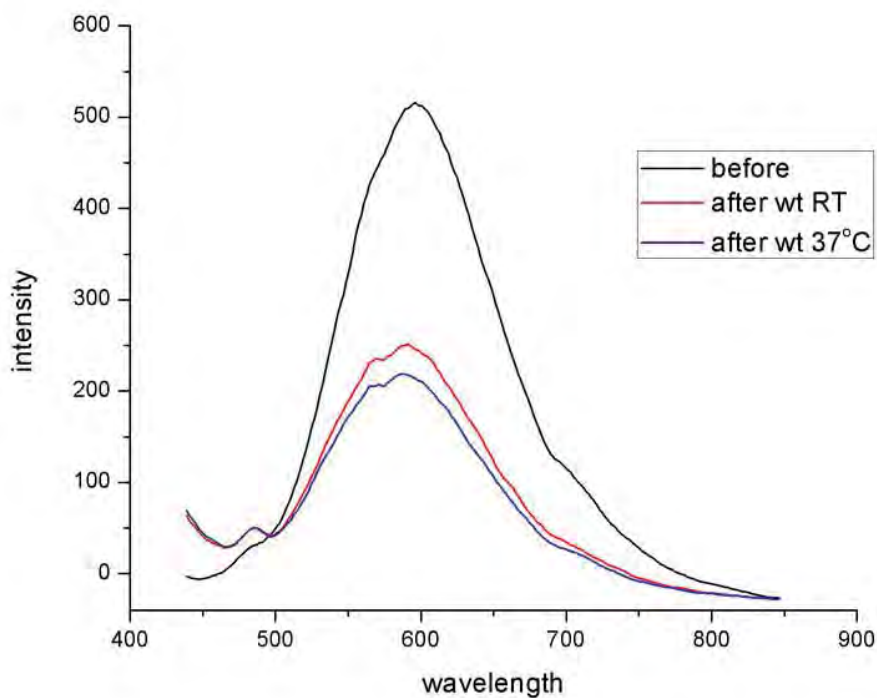


Figure 10 Emission intensity of 20 μ M OB and 2mM of glucose at 609 nm with various unit of glucose oxidase in enzymatic probe system in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

- The studies on the effect of temperature

Moreover, the optimum enzymatic probe systems with different temperature were also explored. **Figure 11** showed the effect of the temperature toward the enzymatic probe system. As expected, a high temperature was able to change emission response better than the room temperature. Therefore, all manipulations of the enzymatic probe system were carried out at 37 °C temperature.



Figur

Figure 11 Emission intensity of 20 μM **OB** and 2 mM of glucose at 609 nm with room temperature and 37°C in enzymatic probe system in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

- The studies on the reaction time

To optimize the reaction time, the fluorescence measurement of the solution of sensor (**OB**), glucose and glucose oxidase was monitored every 5 minutes until 120 minutes. (shown in **Figure 12**) The emission band at 609 nm of **OB** was gradually decreased upon increase of time. However, we chose the reaction time of 60 minutes which is not too long time and the fluorescence quenching is significant change, in optimizing condition of the enzymatic probe system.

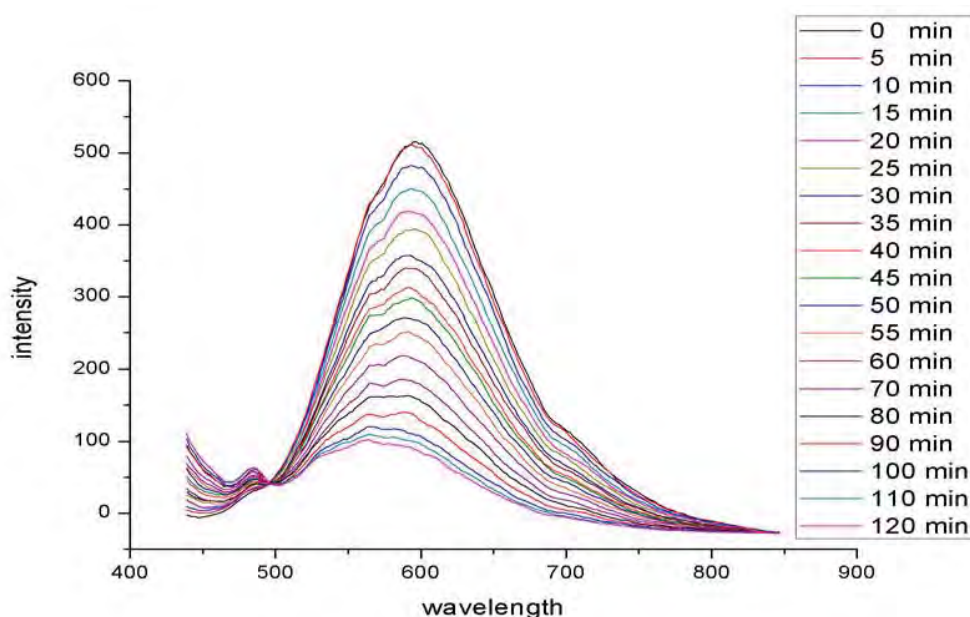


Figure 12 Emission intensity of 20 μM **OB** and 2mM of glucose at 609 nm in enzymatic probe system in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

- Fluorescence measurement of boronic anthraquinone based sensors (OB) for detection of glucose by glucose oxidase in the optimum condition of the enzymatic probe system

To verify the sensing properties of the boronic anthraquinone based sensors (**OB**), the fluorescence titration between **OB** and glucose in the presence of glucose oxidase was investigated in the optimum condition of enzymatic probe system. As depicted in **Figure 13**, the emission band at 609 nm of **OB** was gradually decreased upon the increment of glucose and was approximately constant at high concentration of glucose. To normalize the fluorescence titration of enzymatic probe system of **OB**, the calibration curve was plotted between i_0-i and the glucose concentration (shown in **Figure 14**). The fluorescence response was linear in the range of 0.08 to 0.42 mM with the acceptable correlation coefficient (R^2) of 0.99082 (**Figure 15**). Therefore, the range of concentration for glucose sensing by the enzymatic probe system is 0.08 - 0.42 mM. The limit of detection (LOD) of **OB** for glucose detection is approximately 0.0114 mM or 11 μM .

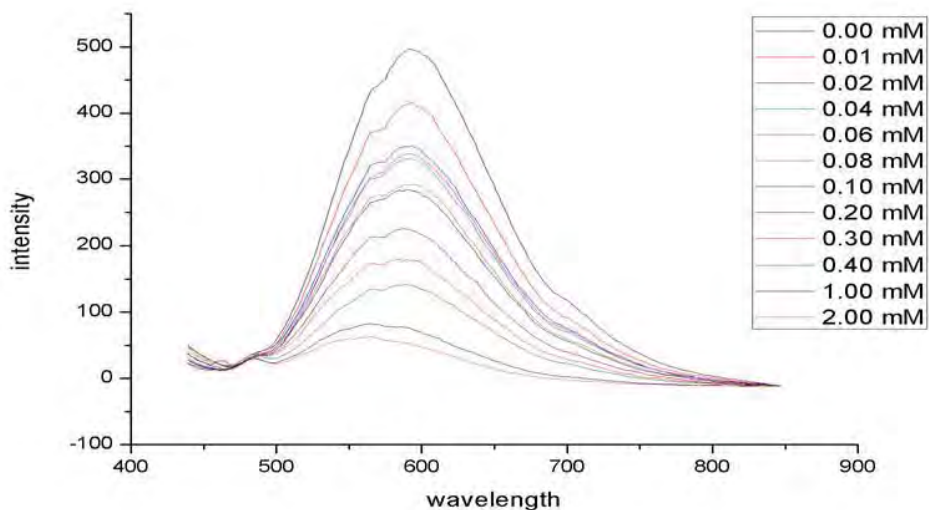


Figure 13 Emission intensity of 20 μM **OB** at 609 nm in enzymatic probe system in 10% DMSO with 0.1M HEPES buffer at pH 7.4.

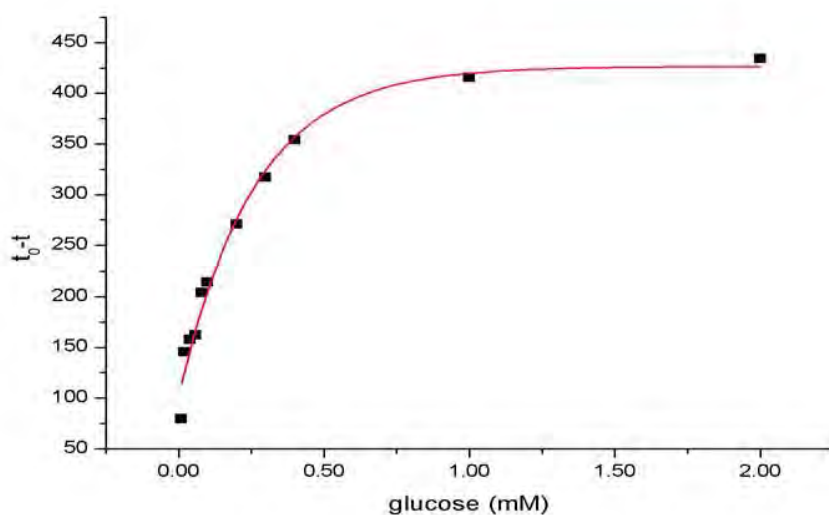


Figure 14 Fluorescence titration curve of 20 μM **OB** and 6 units of glucose oxidase titrated with glucose in 10% DMSO with 0.1M HEPES buffer at pH 7.4. The excitation wavelength was 415 nm. The reaction was performed at 37°C and 1 hour. i_0 is the intensity in the absence of glucose. i is a variation of the emission intensity in the presence of glucose.

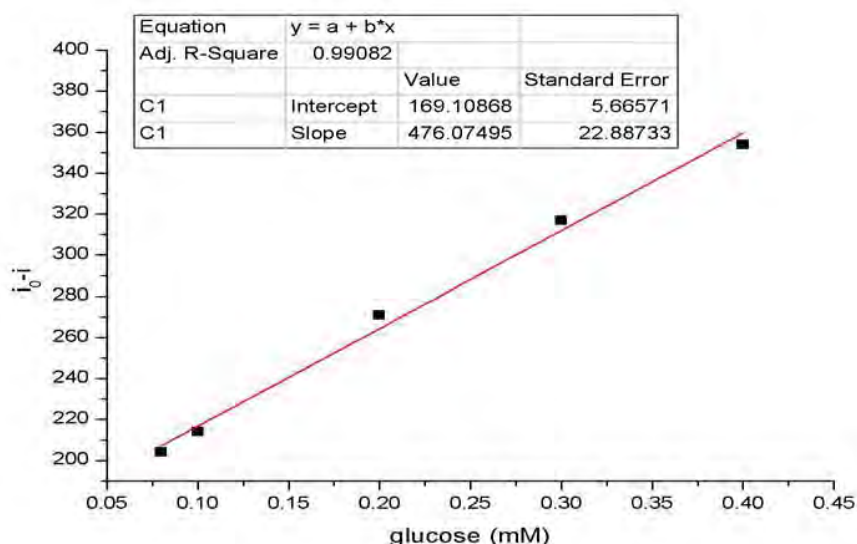


Figure 15 Liner calibration curve of 20 μ M **OB** and 6 units of glucose oxidase titrate with glucose (0.08-0.42 mM) in 10% DMSO with 0.1M HEPES buffer at pH 7.4. The excitation wavelength was 415 nm. The reaction was performed at 37°C and 1 hour. i_0 is the intensity in the absence of glucose. i is a variation of the emission intensity in the presence of glucose.

- Fluorescence measurement of boronic anthraquinone based sensors (OB) in optimum condition of the enzymatic probe system for detection of glucose and other competitive saccharides by glucose oxidase

An important feature of **OB** is to investigate its high selectivity toward glucose over the other competitive saccharides. The effect of other sacharides including D-galactose, D-fructose, D-sucrose and D-maltose were also studied in optimum condition of the enzymatic probe system. As shown in **Figure 16**, the competitive saccarides did not lead to any significant fluorescence changes. Interestingly, the emission spectrum of **OB** was gradually intensified by the addition of fructose. Moreover, for enzymatic probe system in the presence of miscellaneous competitive saccarides and glucose, the fluorescence responses showed the similar change in the case of glucose with **OB** in the enzymatic probe system. Thus, **OB** is indicative of the great selectivity for glucose in this enzymatic probe system.

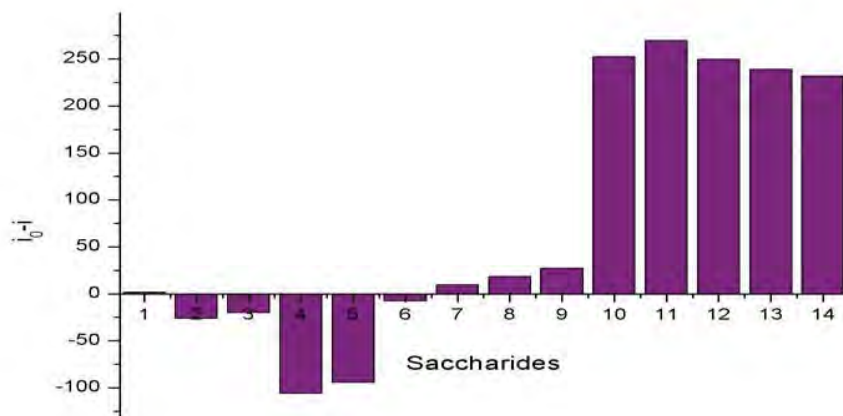


Figure 16 Fluorescence responses of **OB** (20 μ M) to 0.2 mM glucose [1] or to competitive saccharides (2 mM) [2: galactose, 3: galactose + GOx, 4: fructose, 5: fructose + GOx, 6: sucrose, 7: sucrose + GOx, 8: maltose, 9: maltose + GOx] in the presence of another saccharide interference (0.2 mM glucose, 2 mM competitive saccharides) [10: glu + GOx, 11: glu + galactose + GOx 12: glu + fructose + GOx, 13: glu + sucrose + GOx, 14: glu + maltose + GOx].

1.3 Conclusion

We have successfully synthesized the fluorogenic compounds based on boronic anthraquinone (**OB** and **PB**) and glycoside anthraquinone (**OAG** and **PAG**). Firstly, we developed boronic anthraquinone based sensors (**OB** and **PB**) for determining glucose by enzymatic probe system with glucose oxidase using fluorescence spectrophotometry. This method was described that the reaction of glucose oxidase with glucose in the presence of oxygen produced the gluconic acid and hydrogen peroxide which can convert the boronic acid based boronic anthraquinone (**OB**) to be hydroxyl group in 10% DMSO with 100 mM HEPES buffer at pH7.4. The fluorescence response of **OB** was dramatically quenched after adding glucose oxidase in the solution of **OB** and glucose. For **PB**, it was found that it can bind with glucose directly. Thus, it could not be used for glucose sensing via the enzymatic system of glucose oxidase. **OB** is a good candidate for fluorogenic sensor to detect glucose in enzymatic probe system by glucose oxidase. The optimum condition used for enzymatic probe system of **OB** was amount of glucose oxidase at 6 units, the temperature at 37 °C and the reaction time of 60 minutes. The working concentration of glucose in enzymatic probe system of **OB** by glucose oxidase is 0.08 to 0.42 mM.

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Sub-project 2: Self-assembled coordination nanoparticles from nucleotides and lanthanide ions with doped-boronic acid-fluorescein for detection of cyanide in the presence of Cu²⁺ in water

The report in this topic was illustrated according to paper 1 of **Talanta, 2012, 89, 264** as the attached document #1 and #4. And also the extended work in this topic was described as a following:

2.1 Design concept of boronic acid/fluorescein based sensors

Our research group has considerable paid attention on the design, development of new colorimetric sensors, and synthesis of the molecular sensors for cations and anions. Recently, molecular recognition has been performed at the actual heart of sensor chemistry. The process itself includes the interaction between two substances, often defined as a host and guest, a lock and a key or a receptor, sensor and a substrate chemistry. [1] Importantly, recognition events take place through non-covalent interactions comprising hydrogen bonding, ion-dipole interactions, electrostatic interactions, cation- π interactions and π - π stacking interaction. [2] Therefore, the recognition is not just defined as a binding event but also acquired selectivity between the host and guest.

Fluorescein or rhodamine spirolactam derivatives are nonfluorescent and colorless, whereas ring-opening of the corresponding spirolactam enhances in strong fluorescence emission. Recently, a spirolactam ring to ring-opening can be utilized for the detection of metal ions including Hg^{2+} [3], Pb^{2+} [4], Fe^{3+} [5] and Cu^{2+} . [6] Moreover, boronic acid moiety is readily demonstrated to have high affinity for compound which composes of diol groups such as saccharide and carbohydrate, consequently, it has been used as carbohydrate sensors. In addition, Swamy [7] illustrated the utilization of boronic acid-linked fluorescent chemosensors in which the ring-opening process of rhodamine to detect copper ion but there is no precedent exist for the use of boronic acid connected with fluorescein or rhodamine to detect cyanide ion directly.

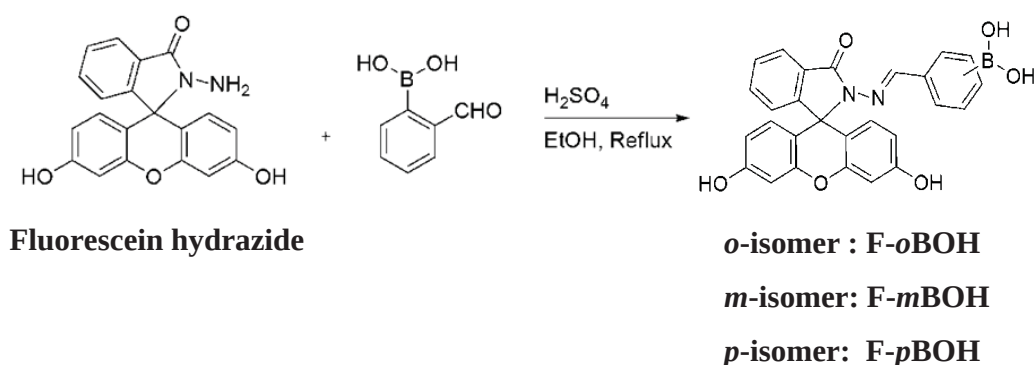
From the reasons mentioned above, we have concentrated on designing the sensors contained both fluorescein and boronic acid moieties. Three sensors with different isomers were synthesized by linking fluorescein hydrazide and formylphenyl boronic acid using the Schiff base reaction pathway illustrated in the scheme 1. All of three sensors consist of copper and cyanide binding sites. We, then, studied the complexation of sensors with copper and cyanide ion in organic solvent using acetonitrile by UV-vis and fluorescence spectrophotometry techniques. Since the sensors contain hydrazone part which can be hydrolyzed by water, therefore, we designed the system to protect the hydrolysis phenomenon by applying adaptive coordination nanoparticles. The complexation of

coordination nanoparticles using several techniques such as UV-vis and fluorescence spectrophotometry, and energy dispersive X-ray (EDX) spectroscopy were also studied.

2.2 Experimental section

- Synthesis of boronic/fluorescein based sensors

- Preparation of sensors **F-oBOH**, **F-mBOH**, and **F-pBOH**



Initially, to prepare the spiro form fluorescein hydrazide by treatment of fluorescein with hydrazine hydrate (yield 60%). In a 100 mL two-necked round bottom flask equipped with a magnetic bar and a reflux condenser, a solution of fluorescein hydrazide (0.173 g, 0.5 mmol) was stirred for 5 min in ethanol (20 mL). Formylphenylboronic acid (0.075 g, 0.5 mmol) in ethanol (30 mL) was added dropwise into the mixture and followed by adding approximately 6 drops of sulfuric acid thereafter the reaction was refluxed overnight under nitrogen atmosphere. Upon completion of the reaction, the solvent was removed under vacuum to obtain a crude product. The crude product was dissolved in ethyl acetate and extracted with water. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The desired products were obtained after recrystallization in hexane/ethyl acetate **F-oBOH** as a white solid 85%, **F-mBOH** as pale yellow solid 92%, and **F-pBOH** as pale yellow solid 90%)

- Characterization of sensors **F-oBOH**, **F-mBOH**, and **F-pBOH**

Characterization data for **F-oBOH**

¹H-NMR (400 MHz, DMSO-*d*₆): δ (in ppm)=9.854 (s, -ArOH, 2H), 9.584 (s, -N=CH, 1H), 8.170 (s, -BOH, 2H), 7.891 (*J*=7.2 Hz, d, -ArH, 1H), 7.609 (*J*=8.0 Hz, dd, -ArH, 2H), 7.755 (m, -ArH, 1H), 7.492 (m, -ArH, 1H), 7.292 (*J*=3.2 Hz, dd, -ArH, 2H), 7.117 (*J*=7.2 Hz, d, -ArH, 1H), 6.644 (s, -ArH, 2H), 6.463 (m, -ArH, 4H).

¹³C NMR (100 MHz, DMSO-*d*₆): δ (in ppm)= 163.446, 158.321, 152.267, 151.063, 150.315, 137.796, 133.748, 129.204, 128.948, 128.836, 127.814, 123.753, 122.987, 112.948, 110.213, 102.447, 65.447;

ESI-HRMS: m/z Calcd for $[M+CH_3OH+H_3O]^+ = 529.1777$, found 529.1542.

Elemental analysis:

Anal. Calcd for C₂₇H₁₉BN₂O₆·1.25H₂O: C, 64.76; H, 4.33; N, 5.59.

Found: C, 64.88; H, 4.04; N, 5.87.

Characterization data for F-*m*BOH

¹H-NMR (400 MHz, DMSO-*d*₆): δ (in ppm)=9.925 (s, –ArOH, 2H), 8.784 (s, –N=CH, 1H), 8.129 (s, –BOH, 2H), 7.918 ($J=7.2$ Hz, d, –ArH, 1H), 7.757 ($J=7.2$ Hz, d, –ArH, 2H), 7.600 (m, –ArH, 2H), 7.474 ($J=8.4$ Hz, d, –ArH, 1H), 7.317 ($J=7.2$ Hz, t, –ArH, 1H), 7.101 ($J=7.6$ Hz, d, –ArH, 1H), 6.666 ($J=1.6$ Hz, d, –ArH, 2H), 6.516 (m, –ArH, 2H), 6.461 (m, –ArH, 2H).

¹³C NMR (100 MHz, DMSO-*d*₆): δ (in ppm)= 163.349, 158.192, 151.597, 150.475, 148.373, 135.726, 133.617, 133.298, 132.947, 128.648, 128.081, 127.509, 126.966, 123.249, 122.816, 111.991, 109.549, 102.163, 64.623;

ESI-HRMS: m/z Calcd for $[M+CH_3OH+H_3O]^+ = 529.1777$, found 529.1552.

Characterization data for F-*p*BOH

¹H-NMR (400 MHz, DMSO-*d*₆): δ (in ppm)=9.924 (s, –ArOH, 2H), 8.952 (s, –N=CH, 1H), 8.110 (s, –BOH, 2H), 7.922 ($J=7.6$ Hz, d, –ArH, 1H), 7.739 ($J=7.6$ Hz, d, –ArH, 2H), 7.620 (m, –ArH, 2H), 7.368 ($J=8.0$ Hz, d, –ArH, 2H), 7.132 ($J=7.2$ Hz, d, –ArH, 1H), 6.645 (s, –ArH, 2H), 6.476 (m, –ArH, 4H).

¹³C NMR (100 MHz, DMSO-*d*₆): δ (in ppm)= 163.805, 158.718, 152.314, 150.587, 148.989, 135.898, 134.737, 134.186, 129.264, 129.017, 128.112, 125.832, 123.927, 123.357, 112.465, 110.237, 102.604, 65.413;

ESI-HRMS: m/z Calcd for $[M+CH_3OH+H_3O]^+ = 529.1777$, found 529.1481.

Complexation Study of F-*o*BOH, F-*m*BOH, and F-*p*BOH in acetonitrile by UV-visible spectrophotometry technique

- Complexation studies of F-*o*BOH, F-*m*BOH, and F-*p*BOH with various cations: copper, cadmium, nickel, silver, zinc, cobalt, and magnesium

Typically, a solution of 1×10^{-3} M of a **F-oBOH**, **F-mBOH**, and **F-pBOH** in 0.01 M tetrabutyl ammonium hexafluorophosphate in dried acetonitrile was prepared. A stock solution of 0.01 M of cation (Cu^{2+} , Cd^{2+} , Ni^{2+} , Ag^{+} , Zn^{+} , Co^{2+} , and Mg^{2+} as trifluoromethanesulfonate and nitrate salt) in dried acetonitrile was prepared in 5 mL volumetric flask. Absorption spectra of **F-oBOH**, **F-mBOH**, and **F-pBOH** with cation complexes were recorded from 200 – 600 nm at ambient temperature. The solution of cation was added directly to 2.00 mL of 3.0×10^{-5} M **F-oBOH**, **F-mBOH**, and **F-pBOH** in a 1-cm quartz cuvette by micropipette and stirred for 5 min. Absorption spectra were recorded after each addition.

- Complexation studies of F-oBOH, F-mBOH, and F-pBOH with copper cation in acetonitrile by UV-visible spectrophotometry

Typically, 0.01 M solution of tetrabutyl ammonium hexafluorophosphate ($[\text{Bu}_4\text{N}][\text{PF}_6]$) (0.194 g) in 50 mL of acetonitrile was prepared. Stock solution of 1×10^{-3} M solution of **F-oBOH** (4.8 mg), **F-mBOH** (4.8 mg), and **F-pBOH** (4.8 mg) in 0.01 M tetrabutyl ammonium hexafluorophosphate in acetonitrile was prepared in a 10 mL volumetric flask. A solution of 5×10^{-3} M of copper trifluoromethanesulfonate in 0.01 M tetrabutyl ammonium hexafluorophosphate in acetonitrile was prepared in a 10 mL volumetric flask.

UV-vis spectra of **F-oBOH**, **F-mBOH**, and **F-pBOH** with copper complexes were recorded from 200-800 nm at ambient temperature. The solution of copper cations was added directly to 2.00 mL of 2.5×10^{-3} M of **F-oBOH**, **F-mBOH**, and **F-pBOH** in a 1-cm quartz cuvette by micropipette and stirred for 2 min. UV-vis spectra were monitored after each addition of cation to gain the ratios of 0:1 to 80:1 for **F-oBOH**, 0:1 to 55:1 for **F-mBOH** and 0:1 to 36:1 for **F-pBOH**.

- Complexation Studies of F-oBOH, F-mBOH, and F-pBOH with copper cation in acetonitrile by fluorescence spectrophotometry

Typically, 0.01 M solution of tetrabutyl ammonium hexafluorophosphate ($[\text{Bu}_4\text{N}][\text{PF}_6]$) in 50 mL of acetonitrile was prepared. Stock solution of **F-oBOH**, **F-mBOH**, and **F-pBOH** in 0.01 M tetrabutyl ammonium hexafluorophosphate in acetonitrile was prepared in a 10 mL volumetric flask. The solution of copper cations was added directly to 2.00 mL of 5×10^{-6} M of **F-oBOH**, **F-mBOH**, and **F-pBOH** in a 1-cm quartz

cuvette by micropipette and stirred for 2 min. Fluorescence spectra were monitored after each addition.

- Determination of detection limit of F-*o*BOH, F-*m*BOH, and F-*p*BOH by Fluorescence spectrophotometry

Typically, 0.01 M solution of tetrabutyl ammonium hexafluorophosphate ([Bu₄N][PF₆]) in 50 mL of acetonitrile was prepared. Stock solution of 1×10^{-4} M of **F-*o*BOH**, **F-*m*BOH**, and **F-*p*BOH** in 0.01 M tetrabutyl ammonium hexafluorophosphate in acetonitrile was prepared in a 10 mL volumetric flask. Fluorescence spectra of **F-*o*BOH**, **F-*m*BOH**, and **F-*p*BOH** (final concentration at 5×10^{-6} M) were monitored for 10 times under the following condition.

Study on the stability of sensor

- The stability of F-*o*BOH in Tris buffer pH 7.0 by fluorescence spectrophotometry

Typically, stock solution of **F-*o*BOH** was prepared in analytical grade ethanol at concentration of 1×10^{-4} M. Stock solution of copper was prepared in MilliQ water at the concentration of 5×10^{-3} M. The solution of **F-*o*BOH** and Tris buffer solution pH 7.0 was mixed together with copper solution to give a final concentration of **F-*o*BOH** at 5×10^{-6} M and copper at 5×10^{-6} M (the ratio of ethanol and Tris buffer was 5:95). Fluorescence spectra were recorded under the following condition by varying time from 0 to 180 minutes

- Study on the stability of F-*o*BOH, F-*m*BOH, and F-*p*BOH by ¹H-NMR spectrophotometry

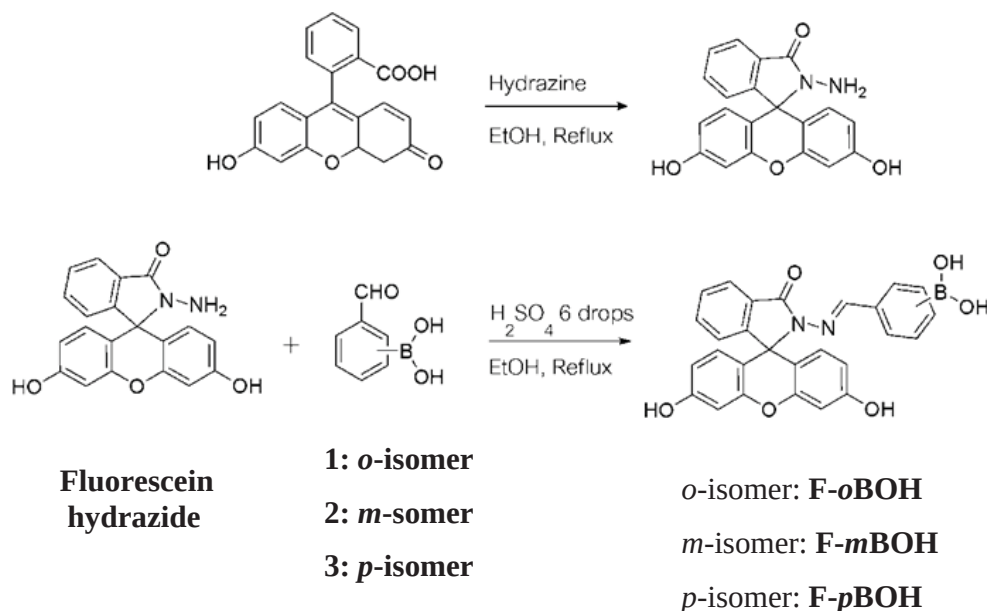
The solution of **F-*o*BOH**, **F-*m*BOH**, and **F-*p*BOH** was prepared in ethanol and Tris buffer pH 8.5 (the ratio of ethanol:buffer solution is 5:95) at concentration 1×10^{-3} M in volumetric flask 5.00. The mixture of **F-*o*BOH**, **F-*m*BOH**, and **F-*p*BOH** stirred at room temperature for 7 days, 2 days and 2 days, respectively.

The mixtures were evaporated under vacuum to remove the remaining solvent. The crude mixtures were dissolved in n-butanol saturated with water and the solution was separated in two layer. The organic layer was collected and dried by vacuum. Finally, the corresponding solution of sensors was prepared for ¹H-NMR experiment by dissolving the sensors in DMSO-*d*₆.

2.3 Results and discussion

- Synthesis and characterization of F-*o*BOH, F-*m*BOH, and F-*p*BOH

Fluorescein hydrazide was selected as a sensory unit due to the different photophysical property of the spirolactam form. The closed form of spirolactam is non-fluorescence while that in opened form shows a strong fluorescence and distinct color change. The synthesis pathway of sensors **F-oBOH**, **F-mBOH**, and **F-pBOH** was illustrated in Scheme 1.



Scheme 1 Synthesis pathway of **F-oBOH**, **F-mBOH**, and **F-pBOH**

Spiro form fluorescein hydrazide can be synthesized by the reaction of fluorescein with hydrazine hydrate. [8] Then, the fluorescein hydrazide was reacted with formylphenylboronic acid using a small amount of sulfuric acid as a catalyst in ethanol under nitrogen atmosphere. The desired product was purified by crystallization applying ethylacetate and hexane as solvents. The $^1\text{H-NMR}$ spectrum of the desired products of **F-oBOH**, **F-mBOH**, and **F-pBOH** was assigned a singlet signal of imine proton ($\text{N}=\text{CH}$) at 9.584, 8.784, and 8.952, respectively. In addition, the $^1\text{H-NMR}$ data of **F-oBOH**, **F-mBOH**, and **F-pBOH** explicated the boronic acid protons ($\text{B}(\text{OH})_2$) at 8.170, 8.129, and 8.110, respectively. The range of aromatic protons (8-6 ppm) of compound **F-oBOH** exhibited a significant difference from fluorescein hydrazide. Furthermore, the $^{13}\text{C-NMR}$ spectra of **F-oBOH**, **F-mBOH**, and **F-pBOH** described a characteristic peak of spirolactam carbon at 65.447, 64.623, and 65.413, respectively. The electrospray high resolution mass spectra confirmed the structure of **F-oBOH**, **F-mBOH**, and **F-pBOH** including methanol and hydronium ion showing the intense peak at m/z 529.1542, 529.1552, and 529.1481,

respectively. According to all results, the obtained products have a structure corresponding to the structure of **F-oBOH**, **F-mBOH**, and **F-pBOH**.

- Complexation Study of F-oBOH, F-mBOH, and F-pBOH in acetonitrile by UV-visible spectrophotometry

- Complexation studies of F-oBOH, F-mBOH, and F-pBOH with various cations: copper, cadmium, nickel, silver, zinc, cobalt, and magnesium

The spirolactam structure of fluorescein ring [9], [4], [10], [11] was a good characteristic to express a strong fluorescence and an obvious color change as a result of cation inducing the ring opening of the spirolactam ring. Initially, we evaluated the complexation abilities of **F-oBOH**, **F-mBOH**, and **F-pBOH** consisted of spirolactam moiety with various cations including Cu^{2+} , Cd^{2+} , Ni^{2+} , Ag^+ , Zn^+ , Co^{2+} , Mg^{2+} in acetonitrile using tetrabutylammonium hexafluorophosphate as supporting electrolyte measured by UV-Vis spectrophotometry technique. In the presence of excess amount of cations, absorption spectra of the sensors in the range of 250 – 600 nm were monitored to explore the selectivity of the sensors.

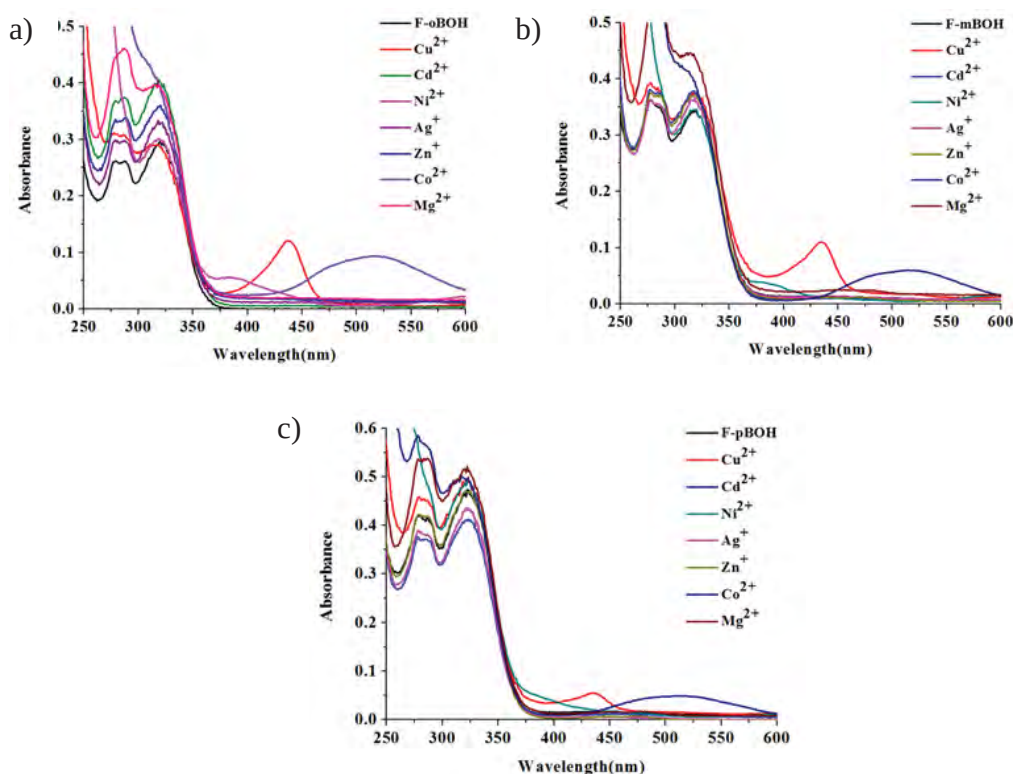


Figure 1 The UV-visible spectra change of a) **F-oBOH**, b) **F-mBOH**, and c) **F-pBOH** (3.0×10^{-5} M) in CH_3CN in the presence of excess amount of various cations.

As shown in Figure 1, the observation of absorption band at 437 nm upon the addition of copper ion in all sensors is indicative of the opened forms of spirolactam ring of **F-oBOH**, **F-mBOH**, and **F-pBOH**. The small changes of the absorption spectra of **F-oBOH**, **F-mBOH**, and **F-pBOH** were observed in the case of Cd^{2+} , Ni^{2+} , Ag^+ , Zn^+ , Co^{2+} , and Mg^{2+} (shown in Figure 1 and 2). This suggested that copper (II) ion can induce selectively a ring opening of spirolactam ring.

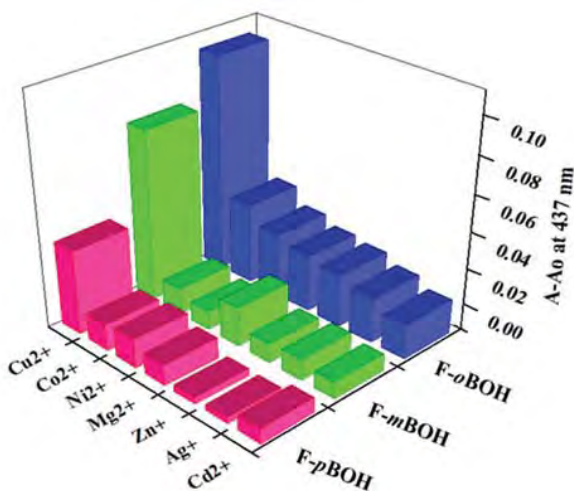


Figure 2 The UV-visible responses ($A-A_0$ at 437 nm) of **F-oBOH**, **F-mBOH**, and **F-pBOH** (3.0×10^{-5} M) in CH_3CN in the presence of excess amount of various cations

-Complexation studies of **F-oBOH**, **F-mBOH**, and **F-pBOH** with copper cation in acetonitrile by UV-visible spectrophotometry

To verify the sensitivity of each sensor, UV-visible titrations of sensors were investigated in acetonitrile using tetrabutylammonium hexafluorophosphate as supporting electrolyte. Prior to UV-visible measurement, the solution mixture of the sensor and copper(II) ion was stirred for 2 minutes. The absorption intensities at 437 nm of the complexation of **F-oBOH**, **F-mBOH**, and **F-pBOH** with copper (II) ion increased as a function of copper(II) concentration as shown in Figure 3a, 3c, and 3e, respectively. The UV-visible titration was measured at least twice. The $\log \beta$ value of **F-oBOH** with copper(II) ion was 6.67 calculated by Spectfit 32 program showing the best fit curve in Figure 3b. Moreover, the $\log \beta$ values of **F-mBOH** and **F-pBOH** with copper(II) ion were 8.87 and 9.88, respectively, calculated by equation 1 showing the best fit curve in Figure 3d and 3f.

$$A = \frac{(A_0 + A_{lim}\beta[guest]^2)}{(1 + \beta[guest]^2)} \quad (1)$$

A = Absorption Intensity of a particular concentration of guest

A₀ = Initial absorption intensity

A_{lim} = Limiting (final) absorption intensity

β = Stability constant of the receptor with the guest

[guest] = Concentration of guest

The log β values of sensors with copper(II) was followed the trend of **F-pBOH** > **F-mBOH** > **F-oBOH**. This sequence was due to the coordination of copper(II) ion with sensors at a boronic acid and a spirolactam ring. The copper(II) ion can bind with **F-pBOH** at the hydroxy group of boronic acid much easier than those of **F-mBOH** and **F-oBOH**, respectively indicated by the highest value of log β of **F-pBOH** with copper(II). Moreover, in the presence of copper(II) ion, the colorless solution of **F-oBOH**, **F-mBOH**, and **F-pBOH** changed to strong yellow color within 3 min, 10 min, and 120 min, respectively. Because, the copper(II) ion can induce the spirolactam ring to be open form of **F-oBOH** easier than those of **F-mBOH** and **F-pBOH** as corresponding to the clearly appearance of the opening spirolactam signal at 437 nm of **F-oBOH**. This suggests that the hydroxy group of **F-oBOH** of boronic acid can chelate with copper(II) ion and induces spirolactam to be open form more quickly than **F-mBOH** and **F-pBOH**.

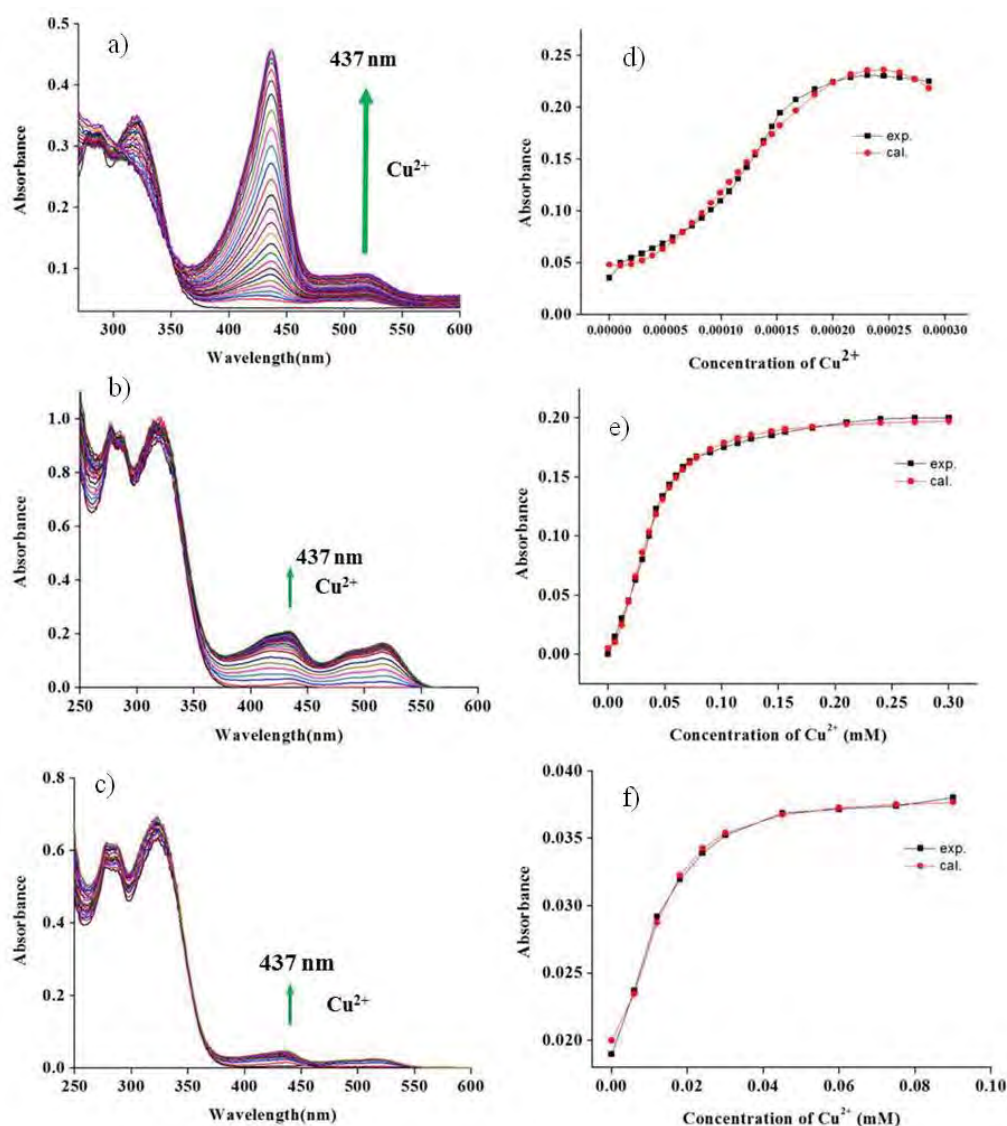


Figure 3 The UV-visible titration spectra of a) **F-oBOH** (2.5×10^{-5} M), c) **F-mBOH** (3.0×10^{-5} M), and e) **F-pBOH** (3.0×10^{-5} M) upon the gradual addition of copper(II) ion in CH_3CN and the compared experiment data and calculated data from UV-visible titration of b) **F-oBOH**, d) **F-mBOH**, and f) **F-pBOH** for calculation of the stability constant.

Herein, the structures of sensors contain two binding sites for copper(II) ion including a boronic part and a spirolactam ring. The association constant of sensors with copper(II) ion were determined by fitting the data of titration curves to a 1:2 binding model using the specfit 32 program and equation 1. In addition, sensor **F-oBOH** was selected to study the stoichiometry of sensors with copper(II) ion using the ESI-HRMS spectroscopy showing the intense peak of $[\text{F-oBOH} + 2\text{Cu}^{2+} + \text{MeOH}]$ at m/z of 629.5241 as shown in Figure 4. It strongly indicated that the stoichiometry of **F-oBOH** with copper(II) ion was

1:2 and corresponded to the binding of copper(II) ion at the spirolactam and boronic acid position shown in scheme 2

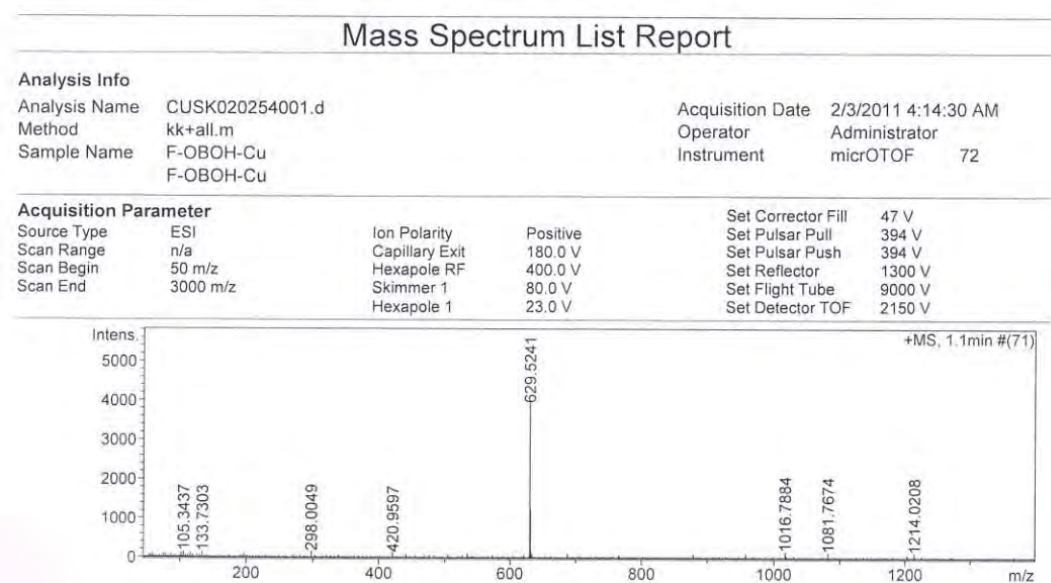
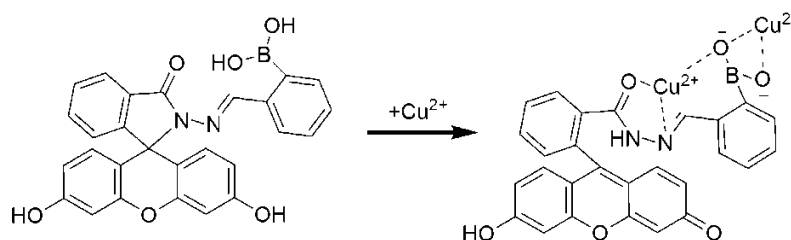


Figure 4 The ESI-High Resolution Mass Spectroscopy (ESI-HRMS) of the complexation between **F-oBOH** and copper(II) ion



Scheme 2 Purpose mechanism of **F-oBOH** complexation with copper(II) ion

Interestingly, our sensors have a boronic moiety which is an electron deficiency boron center that can alternate to be an electron rich by cyanide substitution on the boron atom. To study the cyanide complexation of **F-oBOH**⇌copper(II), the concentration of copper(II) ion of 1.75×10^{-3} M was used to study the cyanide complexation. Upon the addition of cyanide anion, the absorption band at 437 nm of **F-oBOH**⇌copper(II) decreased and turned to the original spectrum of **F-oBOH**. It suggested that cyanide anion can remove copper(II) ion from the binding site.

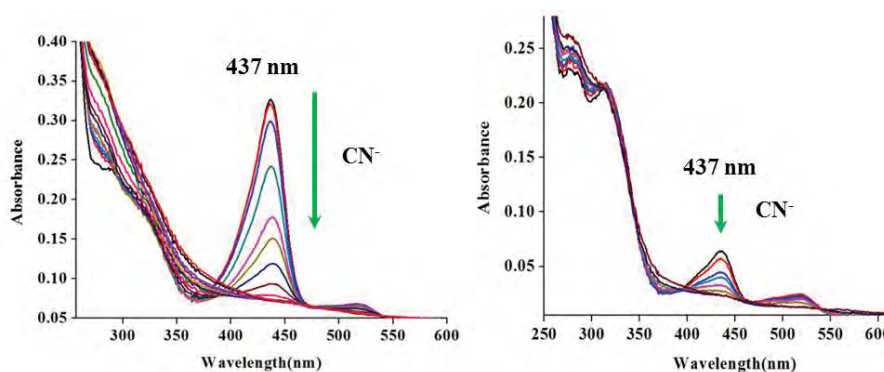


Figure 5 UV-vis spectra of a) **F-oBOH** (2.5×10^{-5} M) b) **F-mBOH** (2.5×10^{-5} M) and Cu^{2+} (1.75×10^{-3} M) in CH_3CN in the presence of different amounts of cyanide anion.

In deeply, the absorption spectrum in the region of 250-325 nm (corresponding to the boron part of **F-oBOH**) is unidentical with the original spectrum of **F-oBOH** (Figure 5). It suggested that the cyanide anion can coordinate at the boron center and the bound copper(II) ion at hydroxy group of boronic acid could not be taken off by the remaining cyanide ion. The binding mechanism was proposed in scheme 3. Furthermore, the observation of the color changes from strong yellow to colorless is consistent with the resultant as shown in Figure 6.

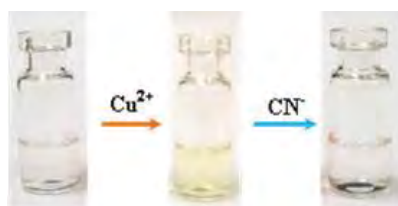
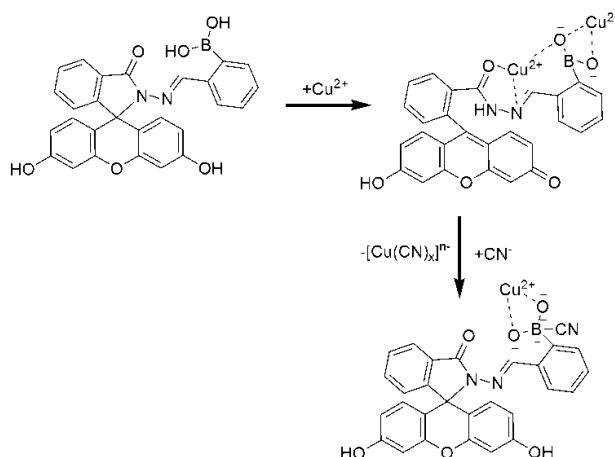


Figure 6 The color changes of **F-oBOH** (2.5×10^{-5} M) after adding and Cu^{2+} (1.75×10^{-3} M) and follows adding CN^- (2.5×10^{-4} M) in CH_3CN .



Scheme 3 Proposed mechanism of **F-oBOH** complexation with copper(II) ion and cyanide anion

-Complexation Study of **F-oBOH**, **F-mBOH**, and **F-pBOH** in acetonitrile by fluorescence spectrophotometry

To verify the sensitivity of each sensor, fluorescence titrations of sensors were examined in acetonitrile using tetrabutylammonium hexafluorophosphate as the supporting electrolyte. Prior to fluorescence measurement, the solution mixture of the sensor and copper ion was stirred for 2 minutes. The fluorescence intensity of the complexation of **F-oBOH**, **F-mBOH**, and **F-pBOH** with copper(II) ion at 475 nm increased significantly as a function of cyanide concentration shown in Figure 7a, 7c, and 7e, respectively. The fluorescence titration was carried out at least twice. The $\log \beta$ values of **F-oBOH** and copper(II) ion are 8.02 calculated by Specfit 32 program showing the best fit curve in Figure 4.7b. Moreover, the $\log \beta$ values of **F-mBOH** and **F-pBOH** are 8.48 and 11.53 calculated by equation 2 showing the best fit curve in Figure 7d and 7f, respectively.

$$I = \frac{(I_0 + I_{lim}\beta[guest]^2)}{(1 + \beta[guest]^2)} \quad (2)$$

I = Intensity of a particular concentration of guest

I_0 = Initial intensity

I_{lim} = Limiting (final) intensity

β = Stability constant of the receptor with the guest

[guest] = concentration of guest

The $\log \beta$ values of **F-oBOH**<**F-mBOH**<**F-pBOH** were observed by both fluorescence and UV-vis techniques. However, the $\log \beta$ values obtained by fluorescence titration are much higher than those obtained by UV-visible titration.

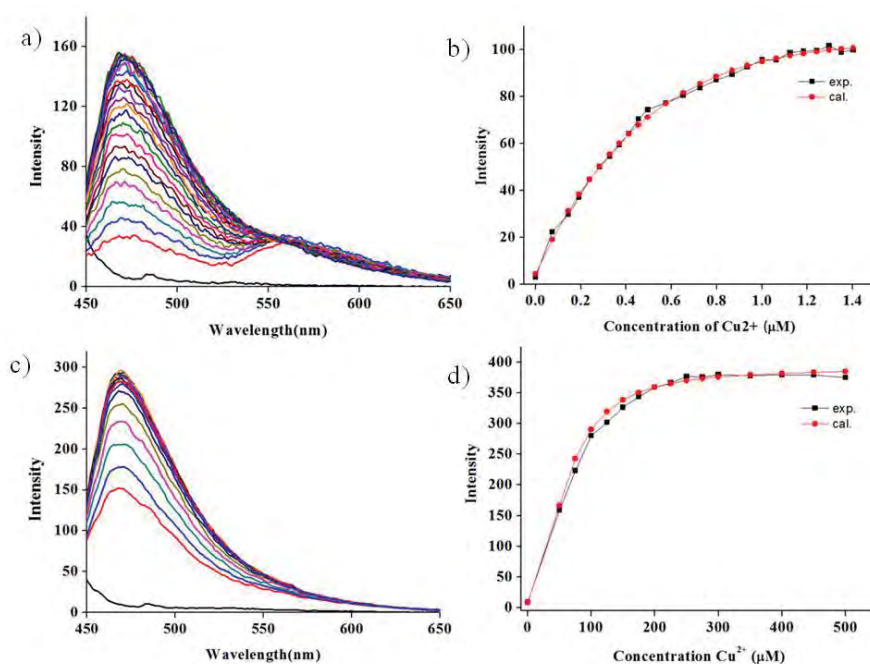


Figure 7 The fluorescence titration spectra of a) **F-oBOH**, c) **F-mBOH**, and d) **F-pBOH** (5×10^{-6} M) upon gradual addition of copper(II) ion in CH_3CN and the compared experiment data and calculated data from fluorescence titration of b) **F-oBOH**, d) **F-mBOH**, and f) **F-pBOH** for calculation of the stability constant. ($\lambda_{\text{ex}}/\lambda_{\text{emiss}} = 437/475$ nm)

- Determination of detection limit of **F-oBOH**, **F-mBOH**, and **F-pBOH** with copper(II) ion by fluorescence spectrophotometry

The detection limit of **F-oBOH**, **F-mBOH**, and **F-pBOH** was evaluated by fluorescence spectroscopy. The 10 samples of **F-oBOH**, **F-mBOH**, and **F-pBOH** (5×10^{-6} M) were prepared and the intensity were measured (Table 1).

Table 1 The intensity of **F-oBOH**, **F-mBOH**, and **F-pBOH** (5×10^{-6} M)

8	16.5390	10.9071	8.3547
9	16.3850	9.5410	9.1373
10	15.9609	10.7878	9.0196
Standard Deviation	0.9507	0.9247	1.3457
1	16.5047	10.5660	10.1678
2	15.1501	9.2396	6.3245
3	16.5699	9.9443	7.2172
4	14.7407	11.0948	6.6163
5	14.0937	11.8388	6.9950
6	14.2172	11.1580	7.3621

The standard deviation of intensity of **F-oBOH**, **F-mBOH**, and **F-pBOH** is shown in Table 1. In addition, the linear relationships of **F-oBOH**, **F-mBOH**, and **F-pBOH** were plotted between the concentration of copper(II) ion and emission intensity at 475 nm shown in Figure 8 and the linear correlation, R^2 , listed in Table 2.

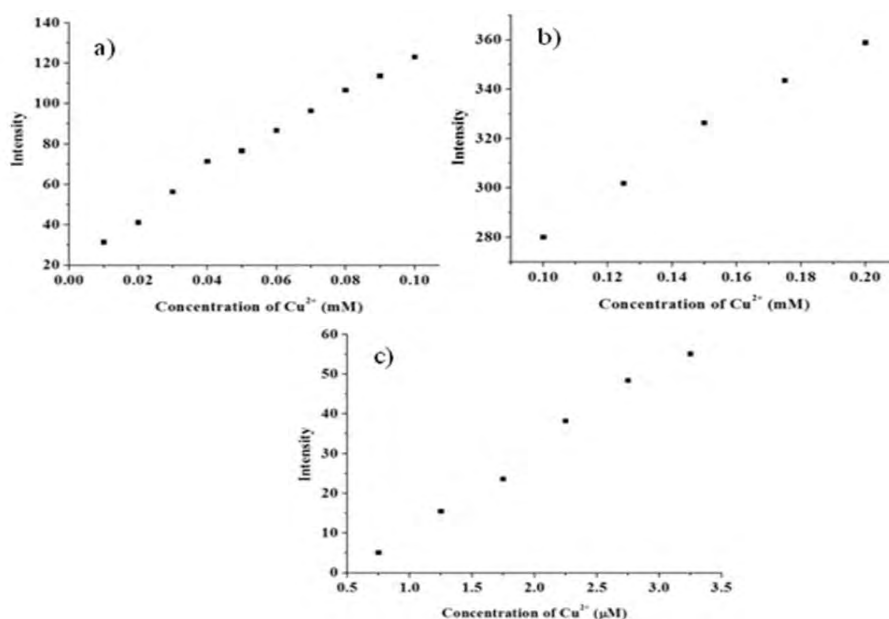


Figure 8 Linear plot between intensity of a) **F-oBOH** b) **F-mBOH** c) **F-pBOH** and concentration of copper(II) ion

The detection limit of three sensors was calculated as three times of the standard deviation of the background noise and shown in Table 2.

Compound	Equation	R^2	Standard Deviation	Detection Limit
F-oBOH	$y = 1 \times 10^6 x + 24.634$	0.9902	0.9507	2.85 μM
F-mBOH	$y = 797492x + 202.47$	0.9912	0.9247	3.48 μM
F-pBOH	$y = 2 \times 10^7 - 10.617$	0.9915	1.3457	1.34 μM

The detection limit indicated that copper(II) ion interact with the hydroxy group of boronic acid at para position better than the meta and ortho position. Moreover, the results also imply that sensors can detect copper(II) ion in the micro molar range.

- Study on the stability of **F-oBOH**

With the aim of developing new chemosensors which can lead to utilize in aqueous solution, therefore, these sensors in aqueous system were investigated. According to the

reporting of Hyman [12], the fluorescein connected to boronate ester cannot bind significantly with any metals due to its instability by hydrolysis in aqueous solution. In this research, **F-oBOH** also consists of the metal binding site and is unstable in aqueous media. The hydrolysis process of **F-oBOH** in the presence of copper(II) ion was investigated by using fluorescence spectrophotometry technique. According to the fluorescence spectra of **F-oBOH** + copper(II) under various time, the fluorescence intensity at 518 nm increased upon the increment of time from 0-180 minutes (shown in Figure 9a, b). This observation is indication of the unstable complex of **F-oBOH** with copper(II) ion in aqueous solution at pH 7.0 as shown in scheme 4.

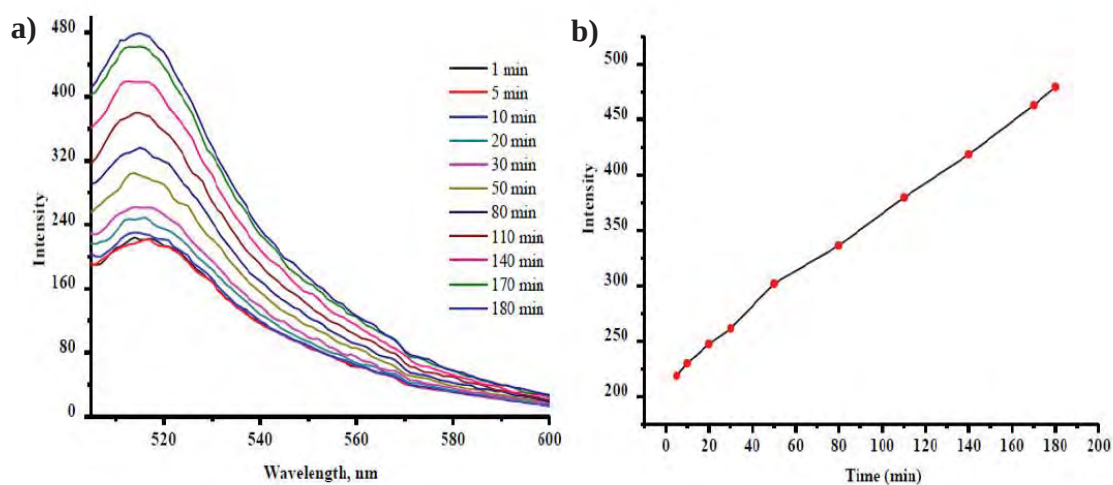
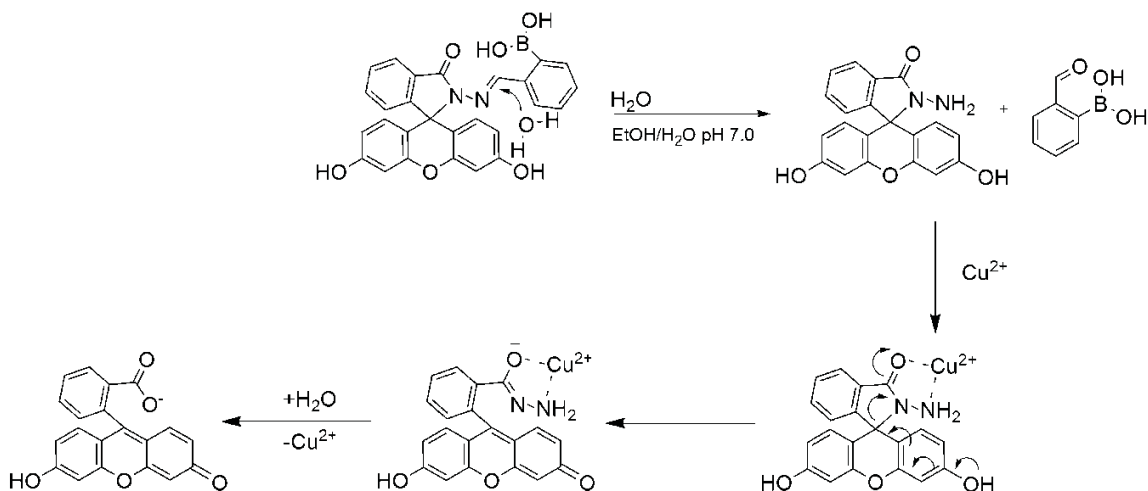


Figure 9 Fluorescence spectra of **F-oBOH** (5 μ M) a) in the presence copper(II) ion (5 μ M) b) The emission intensity at 518 nm in the presence copper(II) (5 μ M) under various time in Tris-HCl 0.01 M pH 7.0 buffer solution (5% EtOH:water), (λ_{ex} = 498 nm)



Scheme 4 Proposed the hydrolysis mechanism of the **F-oBOH** with water followed by reaction with copper(II) to yield fluorescent fluorescein.

With attempts to illustrate the hydrolysis process of sensors, we prepared the hydrolysis product in ethanol and Tris buffer at pH 8.5 which is the suitable pH to generate hydrolysis product in water. According to the $^1\text{H-NMR}$ spectra in Figure 10, the hydrolysis product of **F-oBOH** shows the proton signals at 10.15, 8.25, 7.50 ppm in consistent with $^1\text{H-NMR}$ spectra of 2-formylphenyl boronic acid. However, the **F-oBOH** is still remaining in the reaction. These results strongly support the evidence of the hydrolysis product of **F-oBOH**. Furthermore, the hydrolysis product of **F-mBOH** and **F-pBOH** were evaluated along with the similar method preparation. The results showed that the hydrolysis products of **F-mBOH** and **F-pBOH** are similar to those of **F-oBOH**.

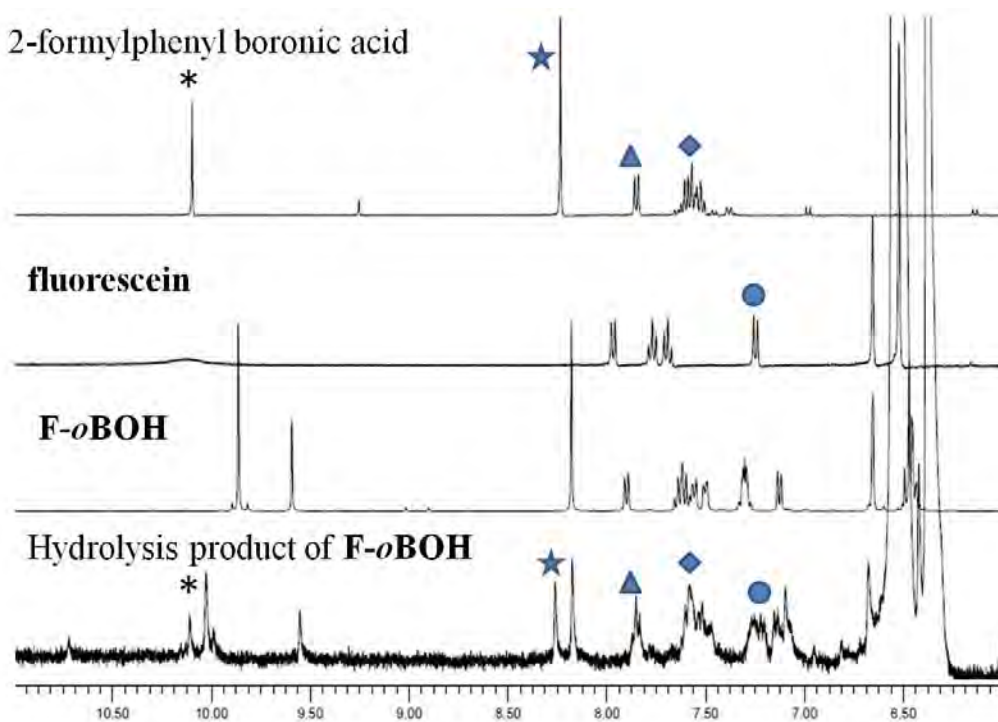


Figure 10 Comparing experiment of hydrolysis product of **F-oBOH** with **F-oBOH**, fluorescein, and 2-formylphenyl boronic acid

2.3 Conclusion

With the recognition of copper and cyanide ions as an objective, we have investigated the synthesis and ion binding properties of boronic sensors and spirolactam ring. In particular, the sensors **F-oBOH**, **F-mBOH**, and **F-pBOH** bearing a boronic acid and a spirolactam ring have been synthesized by the reaction of fluorescein hydrazide and

formylphenyl boronic acid using sulfuric acid as a catalyst. The target sensors were obtained after recrystallization in hexane and ethyl acetate (**F-oBOH** as a white solid 85%, **F-mBOH** as pale yellow solid 92%, and **F-pBOH** as pale yellow solid 90%). Then, all sensors were characterized by using $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and ESI-HRMS spectroscopy. According to the $^1\text{H-NMR}$ spectra of **F-oBOH**, **F-mBOH**, and **F-pBOH** showed a singlet signal of imine proton (N=CH) at 9.584, 8.784, and 8.952, respectively. The $^{13}\text{C-NMR}$ spectra of **F-oBOH**, **F-mBOH**, and **F-pBOH** illustrated a characteristic peak of spirolactam carbon at 65.447, 64.623, and 65.413, respectively. In addition, the ESI-HRMS spectra confirmed the structure of **F-oBOH**, **F-mBOH**, and **F-pBOH** containing methanol and hydronium ion and showed the main intense peak at m/z 529.1542, 529.1552, and 529.1481, respectively. According to all characterization results, the obtained sensors have a structure corresponding to the structure of **F-oBOH**, **F-mBOH**, and **F-pBOH**.

Initially, the **F-oBOH**, **F-mBOH**, and **F-pBOH** were studied on the complexation abilities with several cation including Cu^{2+} , Cd^{2+} , Ni^{2+} , Ag^+ , Zn^+ , Co^{2+} , Mg^{2+} in acetonitrile performed on UV-Vis spectrophotometry. The UV-Vis spectra of **F-oBOH**, **F-mBOH**, and **F-pBOH** displayed the absorption band at 437 nm after adding Cu^{2+} ion, indicating the opened form of spirolactam ring of sensors. This suggested that our sensors have a selectivity toward copper(II) ion. Then, the binding competency of each sensors with copper(II) ion were measured by using UV-vis and fluorescence titration techniques. The $\log\beta$ values evaluated by UV-vis titration of **F-oBOH**, **F-mBOH**, and **F-pBOH** in acetonitrile were 6.67, 8.87, and 9.88, respectively. In comparison, the $\log\beta$ values evaluated by fluorescence titration of **F-oBOH**, **F-mBOH**, and **F-pBOH** in acetonitrile were 8.02, 8.48, and 11.53, respectively. Furthermore, we studied the binding properties of sensors with cyanide anion in acetonitrile by UV-vis spectrophotometry. The results showed that cyanide anion can remove the copper(II) ion from the spirolactam ring of sensor. In contrast, it could not remove copper(II) ion from the hydroxy group of boronic acid but it can substituted on boron center.

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Sub-project 3: Discrimination of nucleotides by a single fluorescence sensor under solvent-dependent recognition patterns and/or multi-component fluorescence sensors

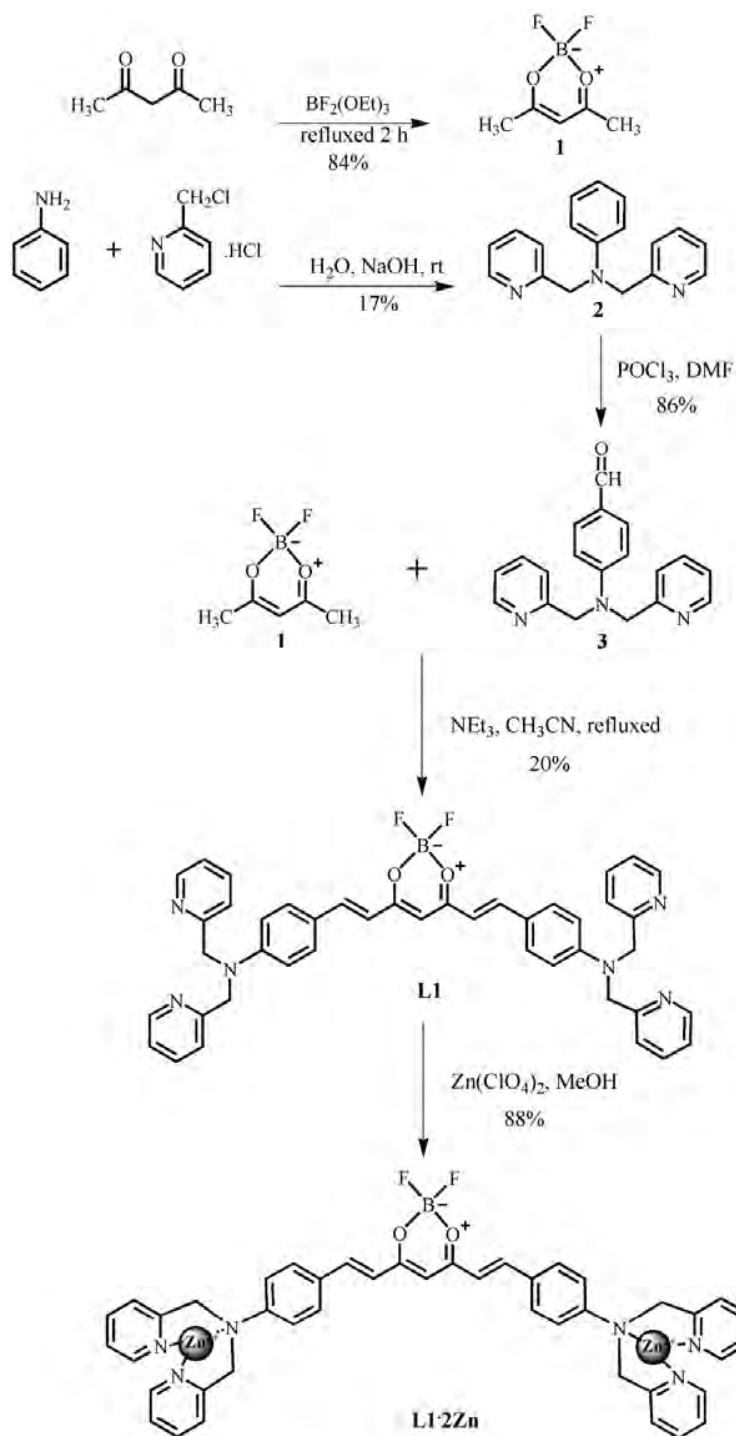
This work has been published in the paper of **Sensor and Actuator B: Chem. 2012** . **2012**, 171– 172, 969–975 (as attached document #2 and #5) and the remaining results were been reported in this reported.

3.1 Design concept

The design and synthesis of chemosensor that was capable to detect cation, anion or nucleotide in aqueous system are attractive approaches. Molecular sensor usually consists of two units: a receptor and a sensory unit. It is well known that dipicolylamine (dpa) is a good receptor unit for binding selectively with Zn^{2+} using lone pair electrons of nitrogen

atom.[1] The sensory units (chromophores and fluorophores) can be attached to dpa. Consequently, these systems are an interest of utilization for recognition of analytes in the applications of molecular switches, molecular sensors, molecular gates, and etc. On the basic of this strategy, we used dipicolylamine moieties for binding transition metals such as Zn^{2+} and Cu^{2+} ion due to their abilities to provide beneficial metal coordination interaction. It was expected for making an electrostatic interaction between cationic Zn^{2+} ion and phosphate species of nucleotides in polar solvents such as water.[2] Actually, the aqueous solvents showed very poor affinity for recognition of anions due to the influence of the strong hydration.[3] Therefore, Zinc(II)-dipicolylamine complexes have been widely used as binding units for phosphate chemosensors.[4-8] Furthermore, BF_2 -curcumin and pyrene are expected to be an excellent fluorophore because BF_2 -curcumin gives a high quantum yield, while pyrene group is very sensitive to solvent polarity and it has widely used for molecular probes using fluorescence spectrophotometry. Consequently, the chemosensor **L1•2Zn** and **L2•Zn** were synthesized by using dipicolylamine as binding sites upon the different fluorophores such as BF_2 - curcumin and pyrene. The complexation was studied by ^1H - NMR, UV-Vis and fluorescent techniques.

The chemosensor **L1** was synthesized as shown in the following scheme 1.



Scheme 1 Synthesis pathway of ligand **L1·2Zn**

-Synthesis and characterization of ligand **L1·2Zn** and **L2·Zn**

- Synthesis and characterization of curcumin derivatives containing dipicolylamine moieties (**L1·2Zn**)

The curcumin derivative containing dipicolylamine moieties (**L1·2Zn**) have been successfully synthesized under the scheme 1. In order to improve the yields of ligand

L1•2Zn, several bases, solvent systems and different reaction conditions were carried out. The ligand **L1•2Zn** was prepared by the nucleophilic substitution, Vilsmeier-Haack and condensation reaction in various conditions. The synthetic pathway was started by modification of acetylacetone with difluoroboron ether under refluxing for 2 h. The pale yellow solid was obtained without the purification in 85% yield.[9] The ^1H -NMR spectrum showed the characteristic peaks of the modified compound as singlet of the methyl protons at 2.29 ppm. The unsaturated proton ($\text{C}=\text{CH}-\text{C}$) moved to downfield shift from 5.46 to 5.99 ppm. Nucleophilic substitution of compound **2** [10] was prepared by the reaction between aniline and 2-chloromethyl pyridine in the presence of sodium hydroxide as base, and the tetrabutylammonium iodide as a phase transfer catalyst in H_2O . The desired product was purified by column chromatography using a gradient of ethyl acetate (20-80%) in dichloromethane as eluent, to afford 17% yield as a white solid of **2**. From ^1H -NMR data of compound **2**, the range of aromatic protons (7.28-6.71 ppm) assigned to aromatic protons of dipicolylamine showed a significant difference from those of aniline. The doublets and triplets of aromatic protons of dipicolylamine moieties were appeared at 8.59 ppm and 7.63 ppm, respectively. Moreover, the appearance of singlet assignment of ArCH_2 at 4.83 in the spectrum supported the structure of compound **2**.

Compound **3** was synthesized by Vilsmeier-Haack reaction.[10] The reaction DMF with POCl_3 produces an electrophilic iminium cation. The slow addition compound **2** into the reaction at 90 °C caused subsequently electrophilic aromatic substitution to generate an iminium ion intermediate, which is easily hydrolyzed by The desired aryl aldehyde **3** was obtained as yellow oil in 86 % yield after neutralized to pH 6-8 with K_2CO_3 to get rid of residue acid and then was extracted with dichloromethane. The ^1H -NMR data of the orresponding **3** displayed the singlet of aldehyde proton at 9.73 ppm. Interestingly, the aromatic protons of compound **3** were assigned by downfield shift to 7.70-6.80 ppm possibly caused by an electron withdrawing group of carbonyl.

Finally, the **BF₂-Curbis(dpa)₂ (L1)** was achieved by condensation reaction [11] between compound **1** and compound **3** in the presence of triethylamine as base in acetonitrile. In order to deprotonate the methyl protons of compound **1**, triethylamine was left stirring in acetonitrile for 1 h prior to addition of the solution of compound **3** in acetonitrile. The reaction was heated at 90 °C under nitrogen atmosphere for 3 days. Obviously, the visual color change of solution was observed from pale yellow to black. The final product (**L1**) was obtained as a dark purple powder in 20% yield after purification by column chromatography using ethyl acetate until 10% methanol as eluent and precipitation with

CH₂Cl₂ and hexane. The ¹H-NMR spectrum of compound **L1** depicted in Figure 1 showed the disappearance of aldehyde proton at 9.73 ppm belonging to compound **3** and that of the chemical shift at 2.99 ppm assigned to methyl group of compound **1**. Furthermore, the aromatic protons shift to upfield at 8.54-6.64 ppm, possibly caused by the influence of an electron donating group of dpa moieties. Interestingly we observed the characteristic signals of the singlet assignment of ArCH₂ at 4.80 and 7.19 ppm in the spectrum which supported the structure of **L1**. IR spectrum of **L1** exhibited an absorption bands of ketone C=O stretching at 1747.83 cm⁻¹, aromatic C=C stretching at 1591.30 and 1513.04 cm⁻¹ and C=C stretching of curcumin at 1382.61, 1173.91 and 1143.99 cm⁻¹. The observation of an intense peak at 718.3061 m/z from ESI-HRMS mass spectrum agreed with the structure of **L1** as shown in the Figure 2. The **L1**•**2Zn** complex was prepared by mixing **L1** with two equivalents of zinc perchlorate in methanol at room temperature. From the ¹H-NMR data as shown in Figure 3 of the desired complex, the aliphatic ethylene protons (NCH₂Ar) of complex was splitted into two double peaks at 4.50 ppm. While the singlet peak of this signal (NCH₂ Ar) at 4.94 ppm belonging to uncomplexed compound still remained in the spectrum. Moreover, the downfield-shifted aromatic protons at 8.86-6.80 ppm stemmed from Zn²⁺ complexed **L1** using lone pair electrons of nitrogen atom.

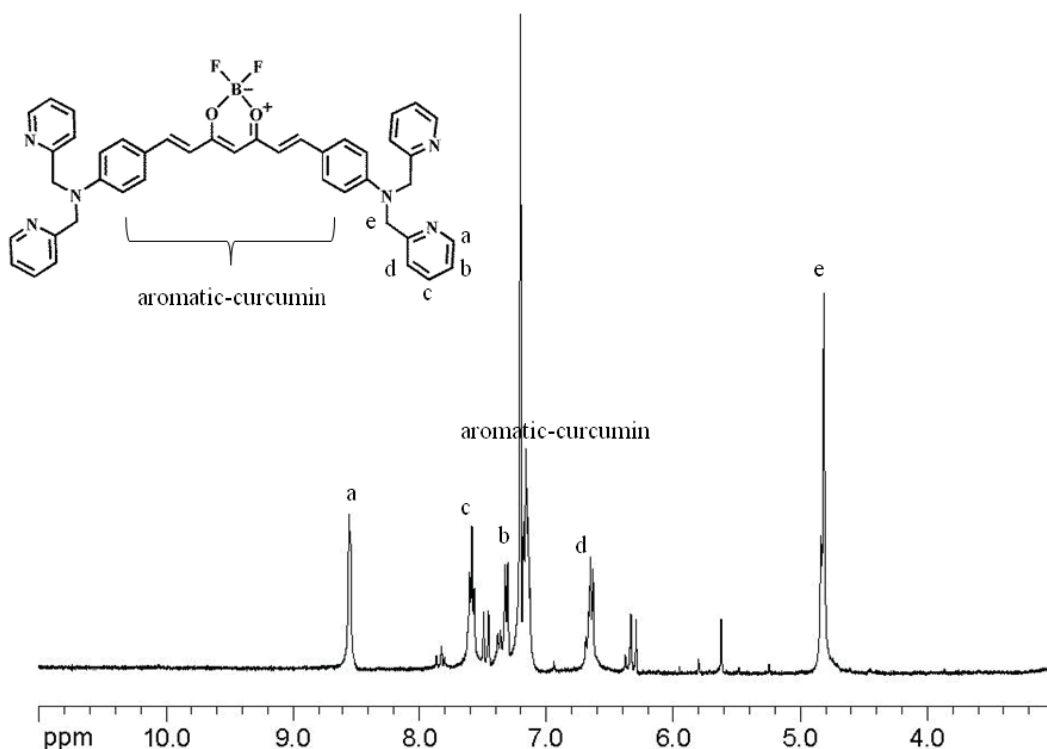


Figure 1 The ¹H-NMR spectrum of curcumin derivatives containing dipicolylamine moieties (**L1**) in CDCl₃ (400 MHz)

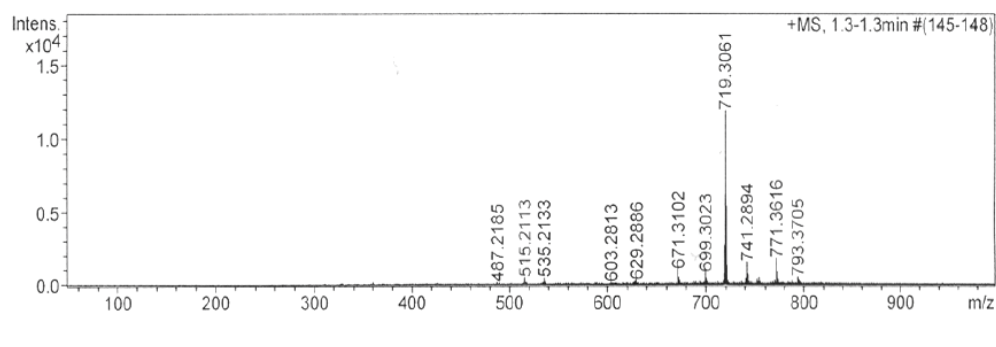


Figure 2 ESI-HRMS mass spectrum of **L1**

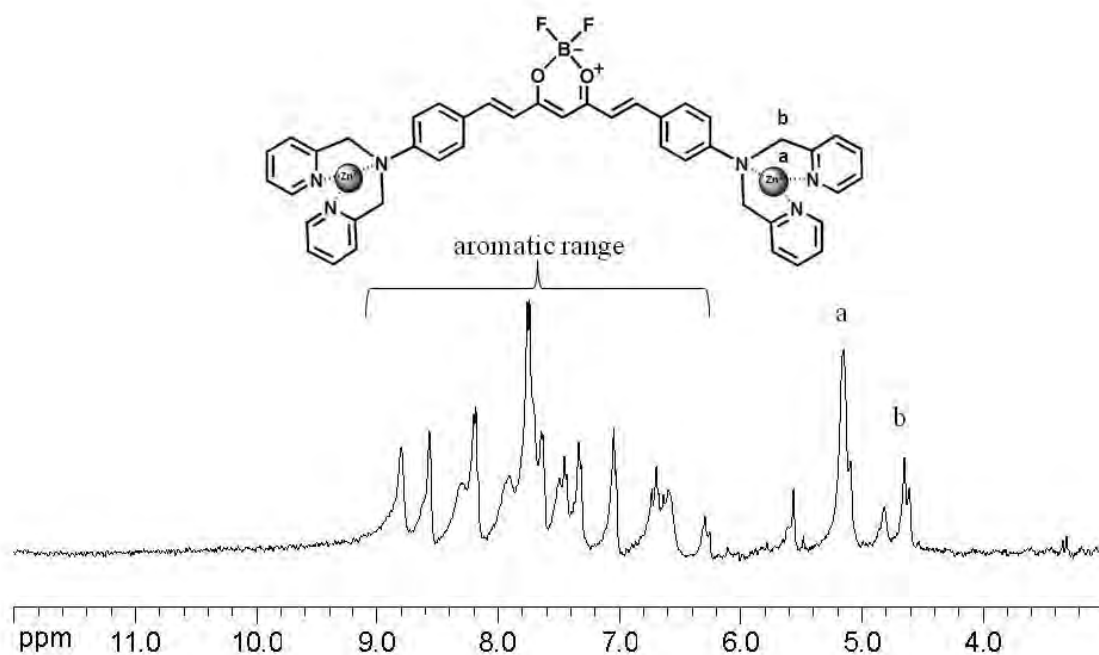


Figure 3 The ^1H -NMR spectrum of curcumin derivatives containing dipicolylamine moieties (**L1**) and Zn^{2+} in CDCl_3 (400 MHz)

-The complexation studies of ligand **L1**

Sensor **L1** also contains the nitrogen donor atoms of the dipicolyl amine moieties, which capable to bind with transition metals such as Zn^{2+} and Cu^{2+} and BF_2 -Curcumin moiety as fluorophore. Thus, we have studied the binding abilities of ligand **L1** toward transition metals by ^1H -NMR, UV-Vis and fluorescent techniques.

- Zn^{2+} ion complexation studies by ^1H -NMR spectroscopy

The binding ability of complexation between sensor **L1** and Zn^{2+} ion were investigated by

^1H -NMR spectroscopy. To reduce the effect of ion pair, perchlorate should be used as counter anion. To evaluate the binding properties, sensor **L1** was titrated with zinc perchlorate monohydrate as shown in Figure 4. ^1H -NMR spectra were found that the chemical shift of the characteristic pyridine protons (NCH) at 8.58 ppm was significantly affected upon zinc complex. After adding 4 equiv. of Zn^{2+} , the signal of aromatic protons of NCH showed a downfield shift from 8.57 ppm to 8.68 ppm. Presumably, lone pair electrons of N atoms bound to Zn^{2+} ions resulting in decrease of electron density. Furthermore, the changes in remaining aromatic protons of ligand were significantly observed. These protons displayed the downfield shift from 7.72-7.26 ppm to 8.289-7.46 ppm, while the protons at conjugate system of curcumin part remained unchanged. It was implied that zinc complex did not affect to the curcumin part. Interestingly, the obvious change of the singlet peak of methylene protons (CH_2) at 4.90 ppm was monitored. The change of these protons demonstrated the splitting of singlet peak into two broad peaks corresponding to the complexation of ligand and Zn^{2+} . [10] Deeply considering ^1H -NMR spectra, no changes of all assignments were observed after adding more than 2 equiv. of Zn^{2+} . This suggested that the complexation of **L1** and Zn^{2+} was completed at 2 equiv. of Zn^{2+} . The chemical shifts of sensor **L1**.

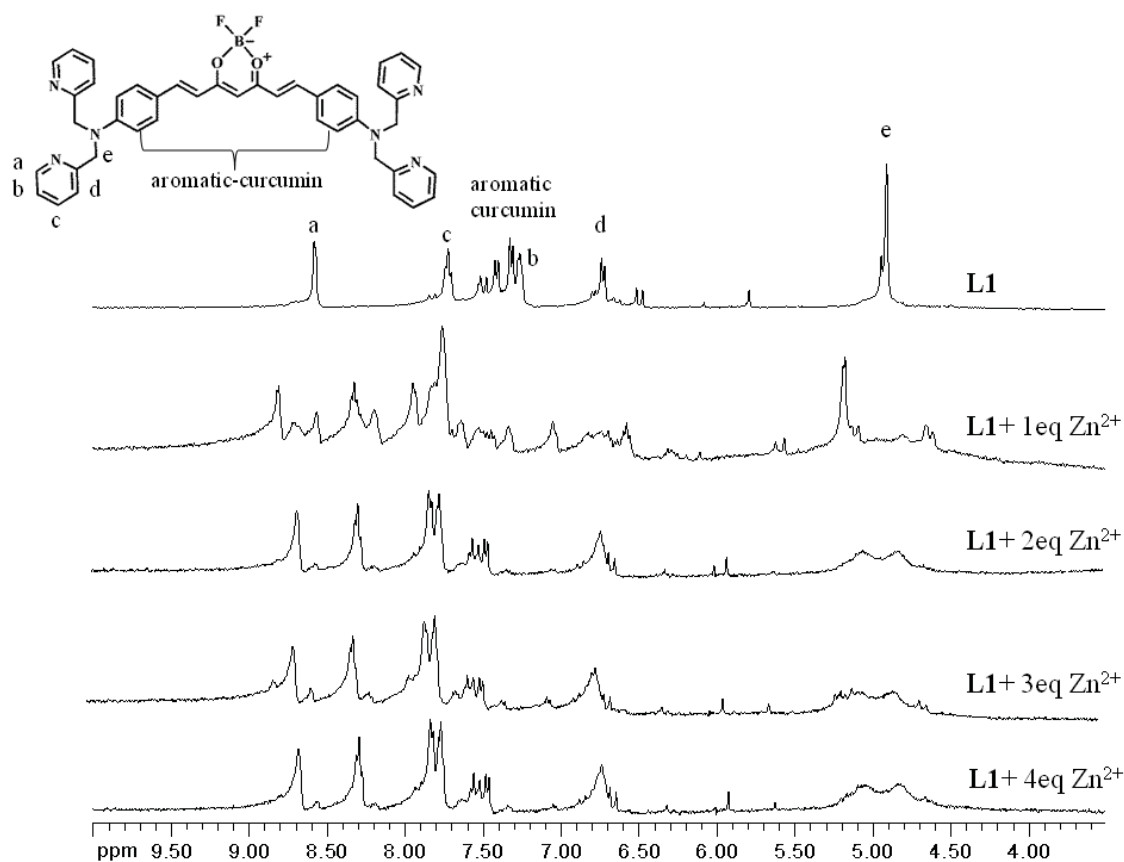


Figure 4 The ^1H -NMR titration spectra of sensor **L1** with Zn^{2+} ion in CD_3CN (400 MHz).

- Cations Zn^{2+} and Cu^{2+} complexation studies by UV-Vis and fluorescence spectrophotometry

Ligand **L1** possess three nitrogen atom of dipicolyl amine moieties as binding sites for Zn^{2+} and Cu^{2+} ion and BF_2 -curcumin is expected to be an excellent fluorophore for cation sensing. The maximum absorption and emission band of ligand **L1** displayed at 488 nm and 587 nm, respectively (spectra illustrated in Figure 5).

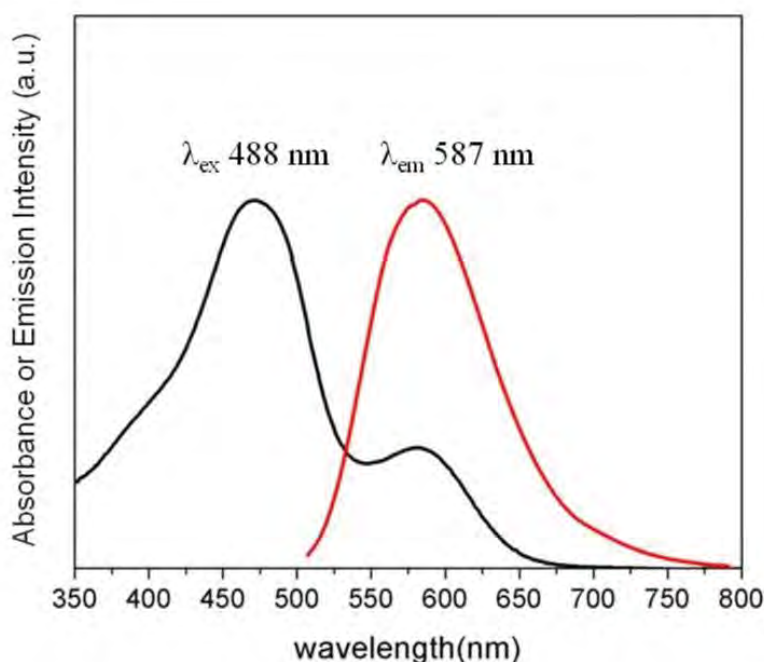


Figure 5 The absorption and the emission spectra of **L1** in acetonitrile.

- Cations Zn^{2+} complexation studies by UV-Vis and fluorescence spectrophotometry

The complexation abilities of ligand **L1** with Zn^{2+} ion have been investigated using the absorption titrations. The Absorption spectra of ligand **L1** (in Figure 6) was changed upon a gradual increase of Zn^{2+} solution. All experiments of absorption titrations were carried out in acetonitrile. The UV-Vis spectra of unbound **L1** showed the absorption band at 472 nm and 581 nm corresponding to the BF_2 -Cur part defined as an acceptor group. The characteristic peak of dipicolyl amine moieties as donor groups displayed a small absorption band at 258 nm. Upon the gradual addition of Zn^{2+} ion to a solution of **L1**, the absorption bands of **L1** at 472 and 581 nm were progressively decreased while a new band at 421 nm was developed. In addition, the absorption band at 258 nm of dpa part was

decreased with a concomitant of a small blue shift. It was indicative of the occurrence of zinc complexed to **L1** at dipicolyl amine parts.[11]68] The blue shift of absorption band at 472 nm and 581nm upon the addition of Zn^{2+} ion can be explained that the electron acceptor ability of the difluoroboron unit was reduced since the lone pair electron on N atom of dpa unit was employed to bind with Zn^{2+} resulting in the suppression of electron delocalization through the conjugate system to the BF_2 -Cur part. [12] Additionally, the visually color change from orange to yellow was markedly observed upon binding with Zn^{2+} (Figure 6 inset).

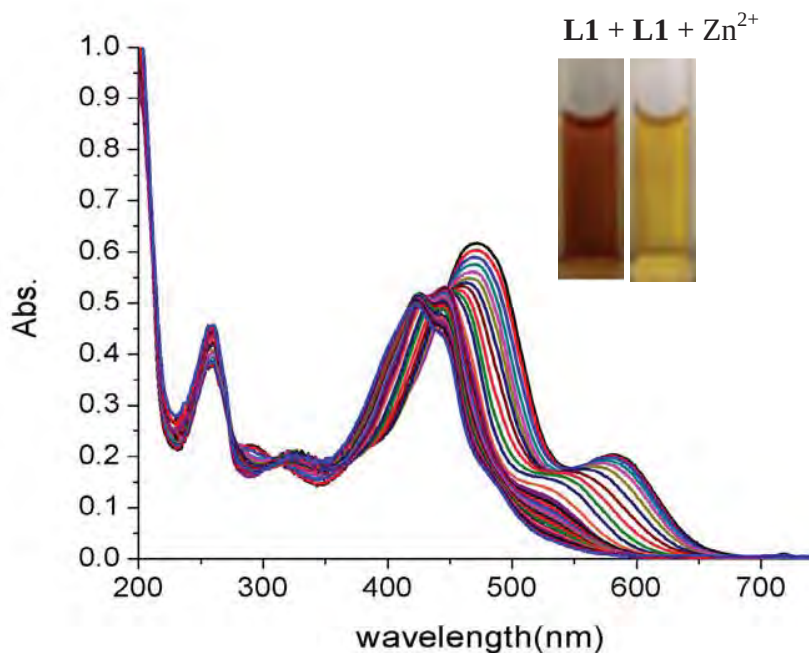


Figure 6 The absorption spectra changes of **L1** (1.80×10^{-5} M) upon the addition of Zn^{2+} (3.60×10^{-4} M), (inset) the color change of **L1** and **L1**+ Zn^{2+} in acetonitrile.

Moreover, the cation complexation ability of ligand **L1** with Zn^{2+} ion was also investigated by the fluorometric titrations in acetonitrile. The fluorescence response of unbound **L1** appeared the characteristic emission band at 587 nm corresponding to fluorophore BF_2 -curcumin. Upon the addition of Zn^{2+} ion, the fluorescence intensity was gradually quenched along with a small bathochromic shift from 587 nm to 615 nm (as shown in Figure 7). The change in the fluorescence spectrum has been attributed to the complexation of Zn^{2+} and **L1**. [12] We proposed that a small bathochromic shift for the complexation of **L1**+ Zn^{2+} was occurred by the substantial suppression of NLCT (spin-allowed amine-to-ligand charge-transfer) character in their emissive states.[13]

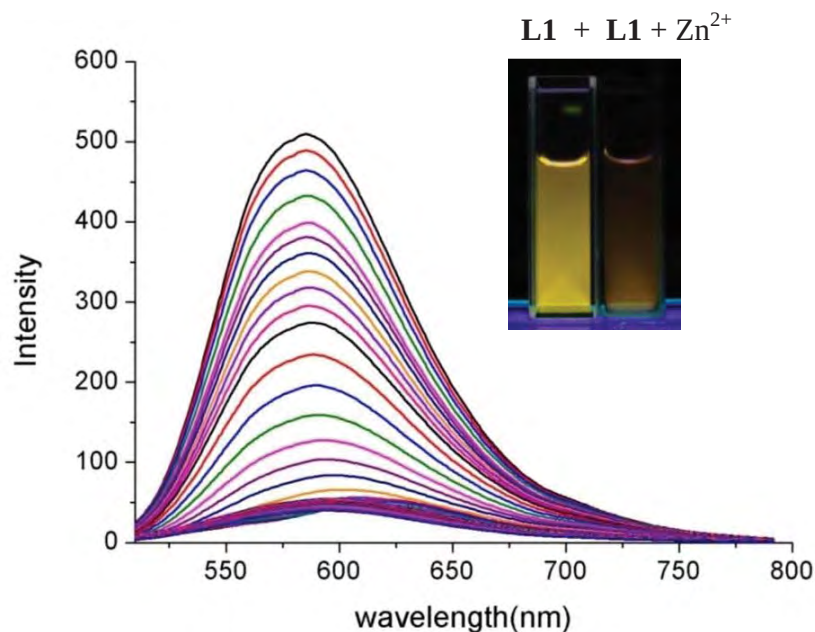


Figure 7 The fluorescence spectrum of **L1** (5×10^{-6} M) in acetonitrile upon the addition of Zn^{2+} (2.00×10^{-4} M), (inset) the color changes of **L1** and **L1** + Zn^{2+} in acetonitrile upon the exposure under UV lamp at 365 nm

The Job's plot analysis was evaluated to determine the binding mode of **L1** and Zn^{2+} using fluorescence technique. A Job's plot showing a maximum point at 0.67 mole fraction indicates that the stoichiometry of host and guest is 1:2 complexes as shown in Figure 8. The fluorimetric titration was performed at least twice and the binding constant of $\text{L1} \cdot \text{Zn}^{2+}$ was determined by SPECFIT32 program. The $\log K$ value of $\text{L1} \cdot 2\text{Zn}^{2+}$ complex was found to be 8.25.

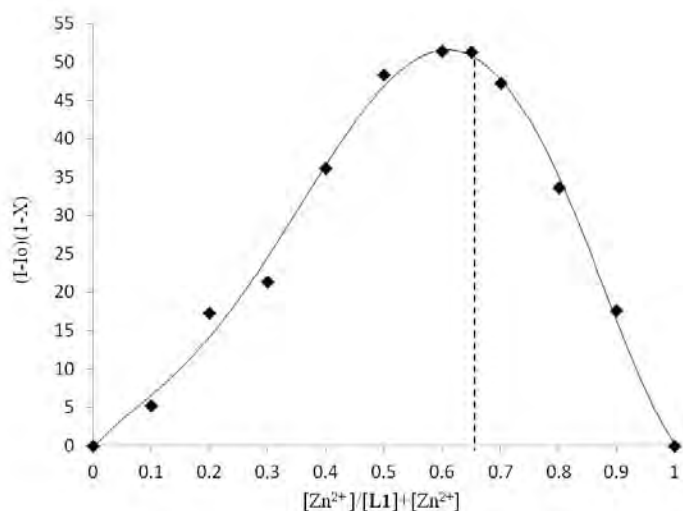


Figure 8 Job's plot of the complexation between **L1** and Zn^{2+} was measured by fluorescence spectrophotometry

The detection limit of Zn^{2+} ion for **L1** was evaluated from the linear plot of fluorescence intensity of **L1**- Zn^{2+} complex at 588 nm versus concentration of Zn^{2+} as shown in Figure 9.

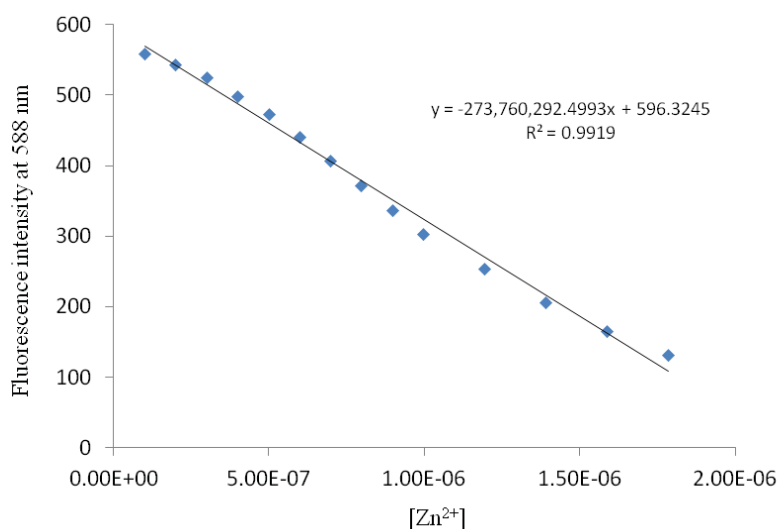


Figure 9 Plot of fluorescence intensity of **L1**- Zn^{2+} complex at 588 nm versus concentration of Zn^{2+} .

In order to further test the interference for other common metal ions on the determination of Zn^{2+} , a competition experiment was performed. The fluorescent probe **L1** in the presence of Zn^{2+} was added by exam of other metal ions. The concentration of **L1** was fixed at 5.0×10^{-6} M in acetonitrile. The fluorescence intensity of **L1** was significantly quenched upon the addition of 10 equiv of Zn^{2+} . Upon the addition of 100 equiv. of various metal ions including Co^{2+} , Ni^{2+} and Cu^{2+} , the fluorescence quenching was remarkably

observed. The case of Ag^+ did not induce the fluorescence change of $\text{L1} \subset 2\text{Zn}^{2+}$ and Cd^{2+} showed a small fluorescence quenching. The relative error of a competition experiment was listed in Table 1. We found that the influence of other transition metals toward the Zn^{2+} sensing of **L1** showed the relative error less than $\pm 10\%$ indicating that **L1** has an excellent selectivity for Zn^{2+} .

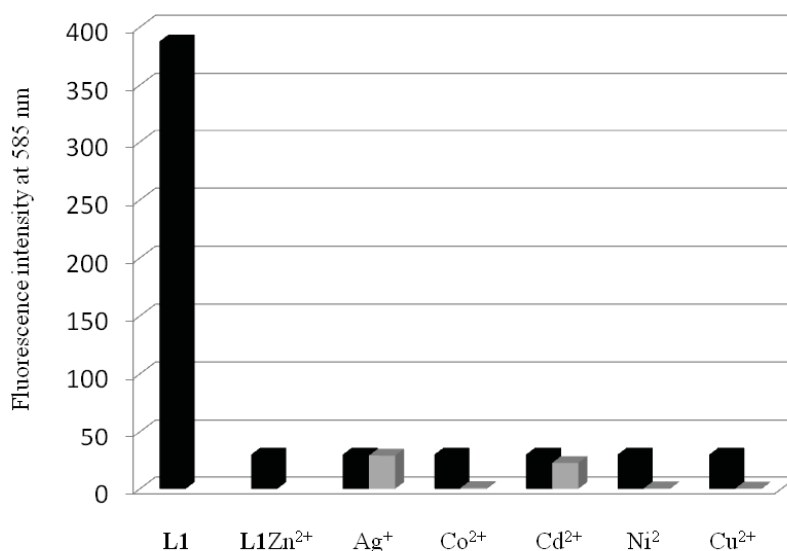


Figure 10 Fluorescence intensity changes of **L1** (5.0×10^{-6} M) in the presence of 10 equiv of Zn^{2+} (back bar) followed by 100 equiv of other cations (gray bar).

Table 1 Interference of several metal ions to the fluorescence response of **L1** with Zn^{2+} (4.76×10^{-5} mol/L)

Interference	Concentration (mol/L)	Fluorescence ($\Delta F = F - F_0$)	Relative error (%), ($\Delta F / F_0 \times 100$)
Ag^+	4.76×10^{-3}	0.84472	-0.24
Co^{2+}	4.76×10^{-3}	-28.7489	-8.03
Cd^{2+}	4.76×10^{-3}	-7.16991	-2.00
Ni^{2+}	4.76×10^{-3}	-29.0731	-8.18
Cu^{2+}	4.76×10^{-3}	-29.1245	-8.13

-Cations Cu^{2+} complexation studies by UV-Vis and fluorescence spectrophotometry

The UV-Vis titration spectra of **L1** toward Cu^{2+} ion were carried out in acetonitrile. Figure 11 displayed the absorption change of **L1** with a gradual increase amount of Cu^{2+} solution. The UV-Vis spectra of unbound **L1** showed the absorption band at 472 nm and 581 nm. Upon the addition of Cu^{2+} ion to a solution of **L1**, the absorption band at 472 and 581 nm decreased gradually and a new band at 421 nm was appeared. Furthermore, the absorption band at 258 nm corresponding to dpa part was progressively developed along with a small blue shift. It suggested that the Cu^{2+} was complexed to **L1** at dipicolyl amine parts. It is noteworthy that the absorption peak *ca.*315 nm is a characteristic peak of Cu^{2+} ion observing from the gradual increase of this peak upon the increment of Cu^{2+} .

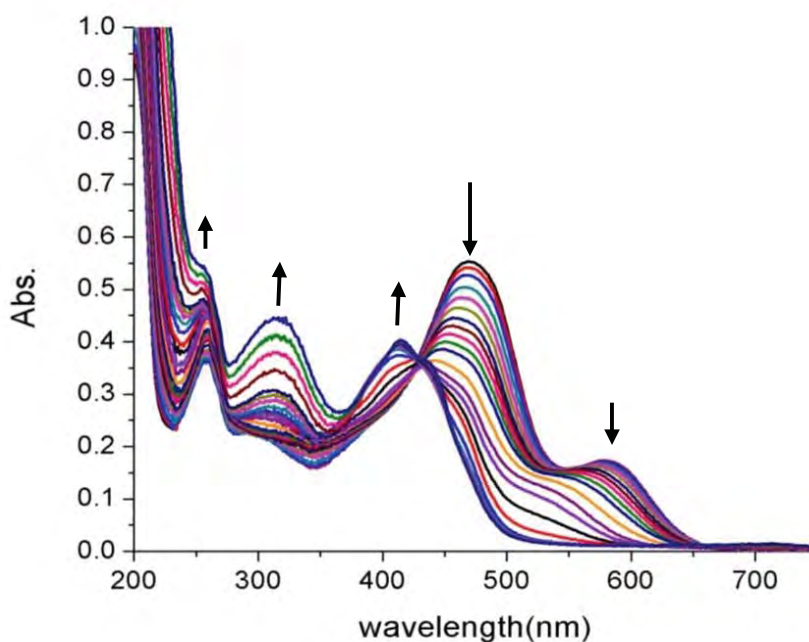


Figure 11 The absorption spectral changes of **L1** (2.00×10^{-5} M) upon the addition of increasing amounts Cu^{2+} (1.00×10^{-3} M)

Considering, the fluorescence responses of **L1** to Cu^{2+} ion by fluorometric titration in acetonitrile exhibited in Figure 12. The characteristic emission band of **L1** appeared at 587 nm. After adding the solution of Cu^{2+} ion, the fluorescence spectra showed a strongly fluorescent quenching at 587 nm with a concomitant of the small blue shift corresponding to the binding of the copper ion to receptor. The fluorescence quenching behavior underwent a PET mechanism which was described that the electron configuration of Cu^{2+} as d^9 was

stabilized by obtaining the electron at excited state of ligand.[14] Consequently, the excited electron could not transfer to ground state resulting in quenching the fluorescent intensity.

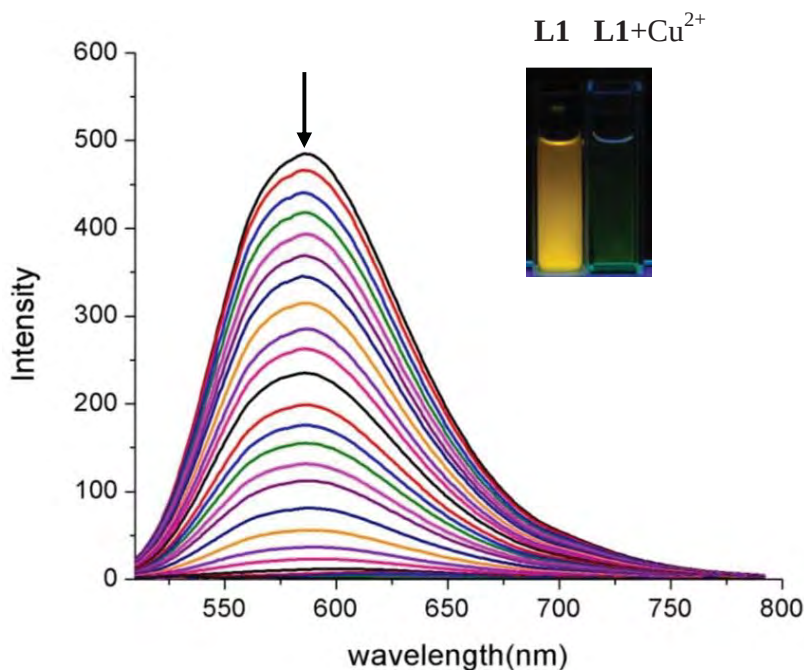


Figure 12 The fluorescence spectrum of **L1** ($5 \times 10^{-6} \text{ M}$) in acetonitrile upon addition of increasing amounts of Cu^{2+} ($2.00 \times 10^{-4} \text{ M}$), (inset) the color change between **L1** and **L1**+ Cu^{2+} in acetonitrile under UV lamp at 365 nm.

The Job's plot analysis for the stoichiometry of **L1** and Cu^{2+} showed the maximum point at 0.67 mole fraction suggesting a 1:2 binding mode (Figure 13). The fluorimetric titration was performed at least twice and the binding constant of **L1** with Cu^{2+} determined by SPECFIT32 program was recorded in Table 2. The log K value of **L1**•**2Cu** complex was found to be 10.52.

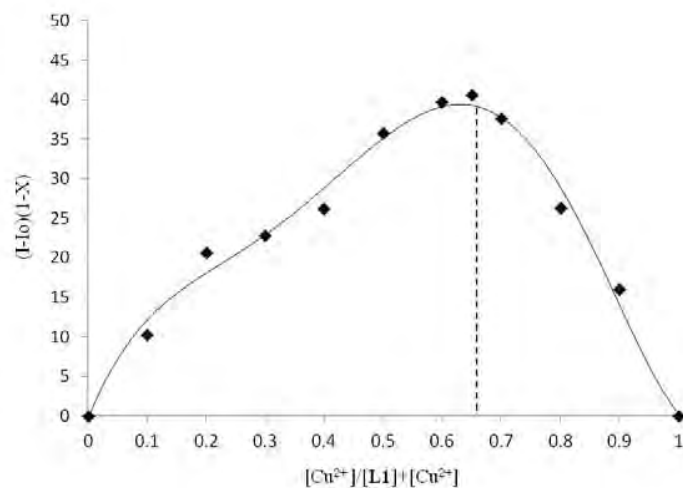


Figure 13 Job's plot of the complexation between **L1** and Cu^{2+} was measured by fluorescence spectrophotometry.

Table 2 The binding constants of ligand **L1** towards Cu^{2+}

first		second		average	
log K	SD	log K	SD	log K	SD
10.53	± 0.04271	10.51	± 0.03016	10.52	± 0.03643

Fluorescence detection limit of Cu^{2+} for **L1** calculated on the basis of $3SD/slope$ was found to be 1.24×10^{-7} M or 7.90 ppb. (Figure 14)

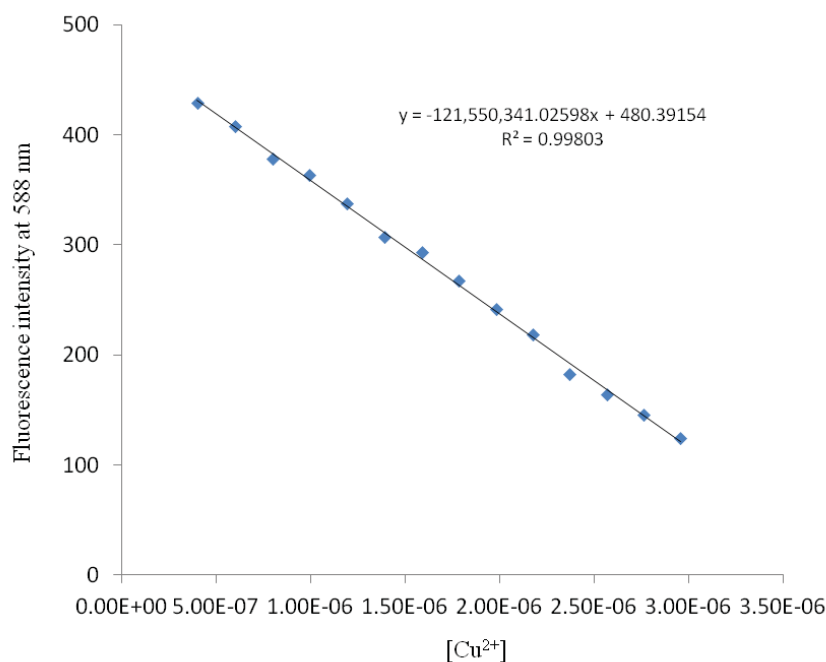


Figure 14 Plot of fluorescence intensity of **L1**·2Cu²⁺ complex at 588 nm versus concentration of Cu²⁺.

The interference of other cations toward Cu²⁺ sensing for **L1** was examined using fluorescence technique (shown in Figure 15). The fluorescence spectra showed no significant change for all other metal ions. The relative errors of a competition of transition metals were collected in Table 3. Thus, **L1** possesses a high selectivity for Cu²⁺ over Zn²⁺ ion.

The relative errors of a competition of the transition metal toward Cu²⁺ detection are less than ±10%. Considering, the influence of other metal ions toward Cu²⁺ sensing for **L1** showed % relative error less than that of other metal ions toward Zn²⁺ sensing. This implied that **L1** preferred to bind Cu²⁺ over Zn²⁺ which agreed with the binding constant results.

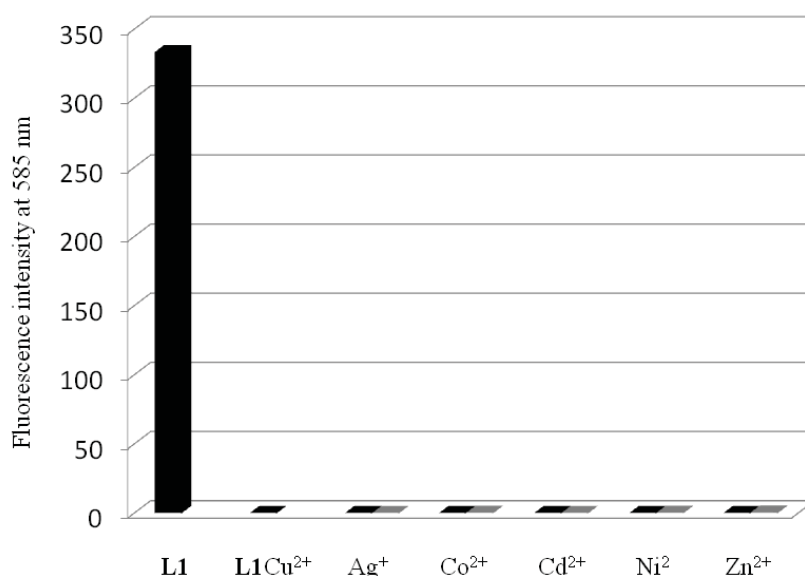


Figure 15 Fluorescence intensity changes profile of **L1** (5.0×10^{-6} M) in the presence of 10 equiv of Cu²⁺ (black bar) followed by 100 equiv of other cations (gray bar).

Table 3 Interference of several metal ions to the fluorescence response of **L1** with Cu²⁺ (4.76×10^{-5} mol/L)

Interference	Concentration (mol/L)	Fluorescence ($\Delta F = F - F_0$)	Relative error (%), ($\Delta F / F_0$) $\times 100$
Ag ⁺	4.76×10^{-3}	0.0202	0.01
Co ²⁺	4.76×10^{-3}	0.2539	0.08
Cd ²⁺	4.76×10^{-3}	0.0220	0.01
Ni ²⁺	4.76×10^{-3}	0.2521	0.07
Zn ²⁺	4.76×10^{-3}	0.4366	0.13

-The complexation study of **L1•2Zn** with nucleotide in aqueous solution

In order to study the binding ability with nucleotide, we used **L1•2Zn** complex is a sensor for detection of nucleotide in aqueous solution. Figure 16 showed the fluorescence change of **L1•2Zn** upon the addition of ATP in HEPES buffer (0.01 M, pH 7.4) and water. The result showed that after adding the solution of ATP in HEPES buffer, the fluorescence spectrum of **L1•2Zn** complex was turned to free ligand **L1** which displayed the

characteristic emission band at 587 nm corresponding of free **L1**. It is indicated that Zn^{2+} ion was removed from **L1** in the water. To gain more information results, the addition of water into the solution of **L1**•**2Zn** complex gave the identical fluorescence response with the free ligand **L1**. The removal of Zn^{2+} ion from **L1** was occurred in water because the lone pair electron of nitrogen atoms at *para* substituted position of benzene ring would delocalize into the conjugate system of $\text{BF}_2\text{-Cur}$ part and subsequent to suppress the binding ability of Zn^{2+} with the N atom adjacent to aromatic ring based curcumine derivatives . Therefore, the binding affinity between Zn^{2+} and two N atoms based pyridine part is very poor. The purposed binding mechanism of **L1** with Zn^{2+} ion was demonstrated in Figure 17.

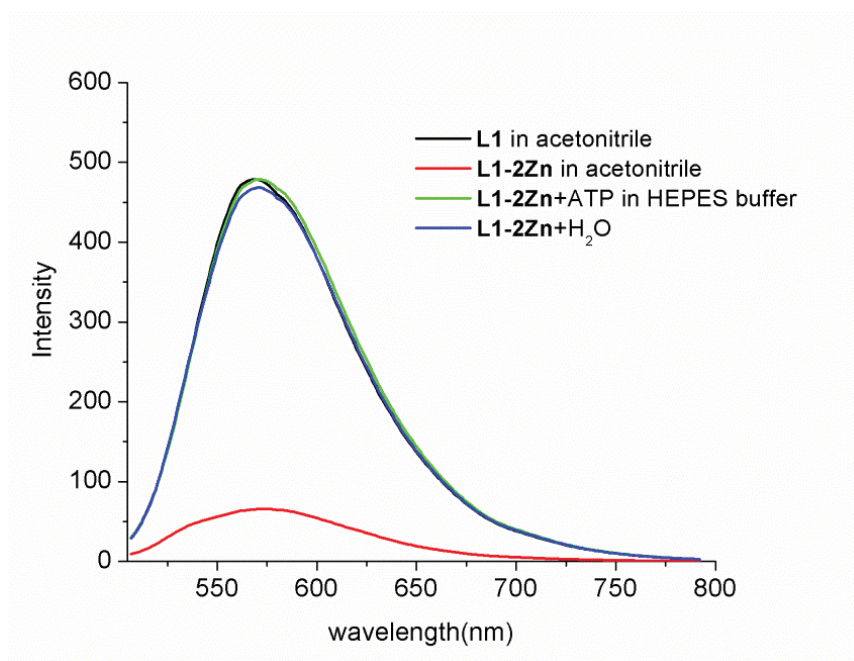


Figure 16 The fluorescence spectral changes of **L1** and **L1**•**2Zn** upon the addition of ATP in HEPES buffer and H_2O .

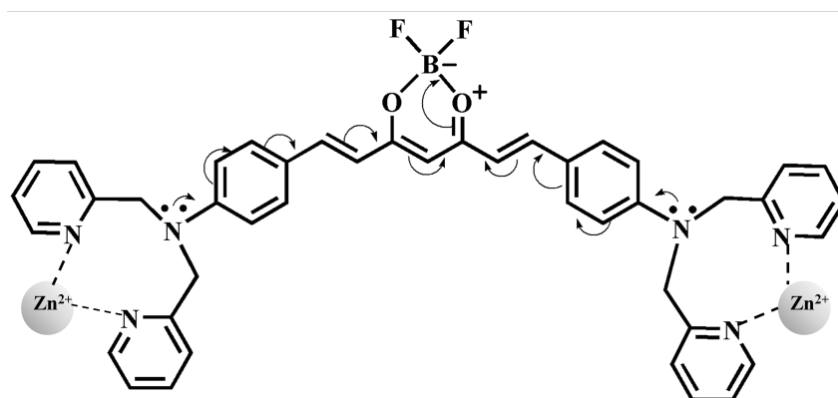


Figure 17 The proposed binding mode of **L1** with Zn^{2+} ion.

However, the use of **L1•2Zn** for nucleotide sensing in aqueous media cannot be studied as a result of Zn^{2+} removal from **L1** in water. Therefore, the **L1•2Zn** complex cannot be applied to nucleotide-sensing purpose in aqueous.

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Moreover, some part of this work entitled “Discrimination of nucleotides by single fluorescence sensor under solvent-dependent recognition patterns has been published in the paper of **Sensor and Actuator B: Chem.** 2012, 171– 172, 969–975 (as the attached document #2). And also, the work related to nucleotide sensing entitled “Discrimination of Nucleotides in Aqueous Solution by the sensor arrays via PCA Method” has been reported in *Srinakharinwirot University (Journal of Science and Technology)*, 2013, 5, 12-23 (as the attached document #5).

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วารสารระดับนานาชาติ

1. Kulchat, S.; Chaicham, A.; Ekgasit, S.; Tumcharern, G.; Tuntulani, T.; Tomapatanaget, B.* “Self-assembled coordination nanoparticles from nucleotides and lanthanide ions with doped-boronic acid-fluorescein for detection of cyanide in the presence of Cu^{2+} in water” *Talanta*, 2012, 89, 264– 269.
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รายชื่อนักศึกษาที่จบการศึกษา

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Self-assembled coordination nanoparticles from nucleotides and lanthanide ions with doped-boronic acid-fluorescein for detection of cyanide in the presence of Cu^{2+} in water

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ABSTRACT

The sensor molecule, **F-oBOH**, containing boronic acid-linked hydrazide and fluorescein moieties was synthesized. For anion sensing applications, **F-oBOH** was studied in aqueous media. Unfortunately, **F-oBOH** was found to be hydrolyzed in water. Therefore, a new strategy was developed to prevent the hydrolysis of **F-oBOH** by applying self-assembly coordination nanoparticles network (**F-oBOH**-AMP/Gd³⁺ CNPs). Interestingly, the nanoparticles network displayed the enhancement of fluorescent signal after adding Cu^{2+} following by CN^- . The network, therefore, possessed a high selectivity for detection of CN^- compared to other competitive anions in the presence of Cu^{2+} . Cyanide ion could promote the Cu^{2+} binding to **F-oBOH** incorporated in AMP/Gd³⁺ CNPs to give the opened-ring form of spirolactam resulting in the fourfold of fluorescence enhancement compared to Cu^{2+} complexation without CN^- . Additionally, the log K value of **F-oBOH**-AMP/Gd³⁺ CNPs toward CN^- was 3.97 and the detection limits obtained from naked-eye and spectrofluorometry detections were 20 μM and 4.03 μM , respectively. The proposed method was demonstrated to detect CN^- in drinking water with high accuracy.

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1. Introduction

Cyanide ion is severely poisonous to living creatures, cellular respiratory system [1]. Nevertheless, the wide applications of cyanide salts have remained prevalent, such as industrial uses [2,3]. Consequently, unintentionally releasing of cyanide into environment should be extremely aware of. The toxicity and beneficial parts of cyanide anion inspired researchers to develop sensors for detection of cyanide anion. The low concentration cyanide detection in water can be explored by various instrumental methods such as ion selective potentiometry [4], voltammetry [5], indirect atomic absorption spectrometry [6], spectrofluorimetry in flow systems [7,8]. Recently, many researchers have an effort to design various chemosensors for CN^- -specific detection. Considerably, the use of this method is beneficial application in terms of inexpensive cost, rapid implementation and providing the low detection limit. For instances, numerous chemosensors in this approach are based on boronic acid derivatives [9–11], Ru(II) containing pyrrolyl quinoxaline [12,13], displacement approach of Cu complex and CN^- [14,15] and amongst other derivatives [16–21].

Nowadays, a number of fluorescein or rhodamine spirolactam derivatives are playing an increasingly important role in the fields of sensing by giving optical changes [22–27]. As widely known in the characteristic of these compounds, the ring-closing form of spirolactam offers nonfluorescent and colorless properties, whereas the ring-opening form enhances a strong fluorescence emission [28–31]. This becomes particularly important when working with a transition metal sensing. The ring-opening form of spirolactam is generated and offers strong fluorescence and color changes. Recently, changing from a spirolactam ring to a ring-opening form can be utilized for the detection of metal ions including Hg^{2+} [32], Pb^{2+} [33], Fe^{3+} [34], and Cu^{2+} [35,36] monitoring the fluorescence enhancement.

Recently, boronic acid moieties have been demonstrated to have high affinities for anions and diol compounds. Yoon and coworkers [37] have developed the fluorescent chemosensors containing boronic acid-linked hydrazide based rhodamine used as Cu^{2+} sensor with respective ring-opening form. Similarly, Franz and coworkers [38] reported the boronic acid-linked hydrazide based fluorescein for detection of Cu^{2+} . Since boronic acid-linked hydrazide group was easily hydrolyzed by Cu^{2+} and water. Boronic acid, therefore, was oxidized by H_2O_2 before the resultant fluorescein hydrazido 2-imidophenol was utilized to detect copper ion with the fluorescence enhancement in organic solvents. To the best

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of our knowledge, the boronic acid moiety in the mentioned sensors was not used to detect other substrates.

Herein, we have developed the systematic methodology to protect the hydrolysis of boronic acid-linked hydrazide group based fluorescein sensors, **F-oBOH**, by applying self-adaptive coordination nanoparticles of nucleotides and lanthanide metal ions. The boronic acid-linked hydrazide based fluorescein sensors, **F-oBOH**, was used as turn-on fluorescence sensor for the selective detection of CN^- in the presence of Cu^{2+} in water. Moreover, we have demonstrated that our proposed method can be used to detect CN^- in drinking water with high accuracy.

2. Experimental

2.1. Instrumentations and materials

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian 400 and Bruker DRX 400 MHz nuclear resonance spectrometers. All of samples were dissolved in deuterated DMSO. The chemical shifts were recorded in part per million (ppm) using a residue proton solvents as internal interference. High resolution mass spectra were determined on Bruker Daltonics DataAnalysis 3.3 with an electrospray ion source using methanol as a solvent. Absorption spectra were measured by a Varian Cary 50 UV-vis spectrophotometer. Fluorescence spectra were performed on a Varian Eclipse spectrofluorometer by personal computer data processing unit. Scanning electron microscopy (SEM) and energy dispersive X-ray (EDS) analysis were performed on a JEOL JSM-6510A with a high resolution of 3.0 nm at 30 kV. Samples were grounded using aluminum stub. Transmission electron microscopy (TEM) was performed on a JEOL JEM 2010 with the field emission gun operated at 200 kV.

2.2. Synthesis

2.2.1. Preparation of fluorescein link boronic acid sensor (**F-oBOH**)

Initially, to prepare the spiro form fluorescein hydrazide by treatment of fluorescein with hydrazine hydrate (yield 60%) [38]. In a 100 mL two-necked round bottom flask equipped with a magnetic bar and a reflux condenser, a solution of fluorescein hydrazide (0.173 g, 0.5 mmol) was stirred for 5 min in ethanol (20 mL). Formylphenylboronic acid (0.075 g, 0.5 mmol) in ethanol (30 mL) was added dropwise into the mixture and followed by adding approximately 6 drops of sulfuric acid. The reaction was refluxed overnight under nitrogen atmosphere. Upon completion of the reaction, the solvent was removed under vacuum to obtain a crude product. The crude product was dissolved in ethyl acetate and extracted with water. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to give a white powder which was then recrystallized in hexane/ethyl acetate affording **F-oBOH** as a white solid in 85% yield. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ (in ppm) = 9.854 (s, $-\text{ArOH}$, 2H), 9.584 (s, $-\text{N}=\text{CH}$, 1H), 8.170 (s, $-\text{BOH}$, 2H), 7.891 (J = 7.2 Hz, d, $-\text{ArH}$, 1H), 7.609 (J = 8.0 Hz, dd, $-\text{ArH}$, 2H), 7.755 (m, $-\text{ArH}$, 1H), 7.492 (m, $-\text{ArH}$, 1H), 7.292 (J = 3.2 Hz, dd, $-\text{ArH}$, 2H), 7.117 (J = 7.2 Hz, d, $-\text{ArH}$, 1H), 6.644 (s, $-\text{ArH}$, 2H), 6.463 (m, $-\text{ArH}$, 4H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ (in ppm) = 163.58, 158.53, 152.41, 150.32, 149.26, 134.47, 133.94, 132.58, 129.08, 128.78, 128.35, 127.88, 126.71, 123.15, 122.31, 111.98, 110.20, 109.96, 102.43, 102.35, 65.37; ESI-HRMS: m/z Anal. Calcd. for $[\text{M}+\text{CH}_3\text{OH}+\text{H}_3\text{O}]^+ = 529.1777$, found 529.1542. Anal. Calcd. for $\text{C}_{27}\text{H}_{19}\text{BN}_2\text{O}_6 \cdot 1.25\text{H}_2\text{O}$: C, 64.76; H, 4.33; N, 5.59. Found: C, 64.88; H, 4.04; N, 5.87.

2.2.2. Preparation of **F-oBOH** doped nucleotide/lanthanide CNPs (**F-oBOH-AMP/Gd³⁺** CNPs)

Coordination nanoparticles were prepared by mixing aqueous mixture of AMP (10 mM, 1.0 mL) and **F-oBOH** (0.2 mM, 0.5 mL) and followed by adding the ethanol solution of $\text{Gd}(\text{NO}_3)_3$ (10 mM, 0.5 mL) into this mixture at room temperature. Finally, the volume of solution was adjusted to be 10 mL by 0.1 M HEPES buffer pH 7.4. Immediately, the colorless precipitate nanoparticles were observed. After stirring the mixture for 1 h, the resultant aqueous suspensions were washed with MilliQ water several times and gathered by ultracentrifugation. Accordingly, the coordination nanoparticles were dispersed in water by sonication. The prepared nanoparticles were examined by SEM, EDS and TEM measurements.

2.3. Complexation studies

2.3.1. Complexation studies of **F-oBOH-AMP/Gd³⁺** CNPs $\subset$ copper(II) with cyanide anion by fluorescence spectrophotometry

All fluorescent experiments were measured in aqueous solution which was prepared by 0.1 M solution of HEPES buffer (pH 7.4) in MilliQ water with sodium chloride as supporting electrolyte. Initially, a 47 μL of 2.15×10^{-3} M copper(II) nitrate was added directly to 2.00 mL of 1×10^{-5} M **F-oBOH-AMP/Gd³⁺** CNPs in a 1-cm quartz cuvette by micropipette and stirred for a minute. A stock solution of sodium cyanide (2×10^{-3} M) was prepared in HEPES buffer (pH 7.4) and then was added in portion via micropipette (10–80 μL) to a mixture solution of **F-oBOH-AMP/Gd³⁺** CNPs and copper cation. After each addition, the mixture was stirred and the fluorescence spectra were recorded.

2.3.2. Determination of detection limit of **F-oBOH-AMP/Gd³⁺** CNPs $\subset$ copper(II) with cyanide anion

Typically, 0.1 M solution of HEPES buffer (pH 7.4) was prepared in MilliQ water with sodium chloride as supporting electrolyte. Stock solution of 1×10^{-5} M of **F-oBOH-AMP/Gd³⁺** CNPs was prepared in volumetric flask (25 mL). A solution of 2.15×10^{-3} M copper(II) nitrate was prepared in MilliQ water in a 10 mL volumetric flask. Fluorescence spectra of **F-oBOH-AMP/Gd³⁺** CNPs with copper (final concentration at 5×10^{-5} M) complexes were monitored for 10 times and all data were used to calculate the detection limit. For naked-eyed detection limit, in each portion, the solution of copper cation (47 μL) was added directly to 2.00 mL of 1×10^{-5} M **F-oBOH-AMP/Gd³⁺** CNPs in each vial by micropipette and stirred for a minute. The stock solution of cyanide anions (2×10^{-3} M) was added in portion (20–400 μL) to each vial of the mixture solution of **F-oBOH-AMP/Gd³⁺** CNPs and copper cation.

2.3.3. Interference studies by fluorescence spectrophotometry

Typically, 0.1 M HEPES pH 7.4 was prepared in all interference studies. In the cuvette, the solution of 1×10^{-5} M **F-oBOH-AMP/Gd³⁺** CNPs (2 mL) and 1×10^{-5} M Cu^{2+} and 1×10^{-4} M CN^- was added by the miscellaneous anions including F^- , Cl^- , Br^- , I^- , OH^- , NO_3^- , ClO_4^- , BzO^- , H_2PO_4^- and SCN^- (the amount of foreign anion according to in Table 1). The emission spectra were recorded after stirring for 5 min.

3. Results and discussion

3.1. Synthesis and characterization of sensory molecules

We have synthesized the sensor, **F-oBOH**, by attaching boronic acid and hydrazide linker to fluorescein for detection of cyanide in the presence of Cu^{2+} . The synthetic procedure of **F-oBOH** is illustrated in Scheme 1. Fluorescein hydrazide was prepared by the addition of hydrazine into the carboxylic moiety of fluorescein

Table 1
Effect of interference anions on the determination of CN^- ($\text{C}_{\text{CN}^-} = 24.5 \text{ ppm}$).

Foreign anion	Equivalent	%Relative error	Interference (M)	Tolerance limit (ppm)
Cl^-	1000	0.620	0.5000	29,220
Br^-	1000	1.248	0.5000	51,450
NO_3^-	1000	1.300	0.5000	42,505
ClO_4^-	1000	3.610	0.5000	70,230
I^-	100	7.160	0.0500	7495
SCN^-	100	8.740	0.0500	4859
BzO^-	100	4.125	0.0500	7206
OH^-	10	6.743	0.0050	200
F^-	5	9.514	0.0025	145
H_2PO_4	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a

^a Not determine.

[13]. The desired product was accomplished by coupling fluorescein hydrazide with 2-formylphenylboronic acid in the presence of sulfuric acid (catalyst) followed by refluxing in ethanol under nitrogen atmosphere to afford the imine group linked to the boronic acid unit. **F-oBOH** was obtained as a white solid in 85% yield. The ^1H NMR spectrum of the **F-oBOH** showed a singlet signal of imine proton ($\text{N}=\text{CH}$) at 9.584 ppm and the boronic acid protons ($\text{B}(\text{OH})_2$) at 8.170 ppm. The ^{13}C NMR spectra of **F-oBOH** showed a characteristic peak of spirolactam carbon at 65.447 ppm. In addition, the electrospray high resolution mass spectrum supported the structure of **F-oBOH** showing the intense peak at m/z 529.1542 ($[\text{M}+\text{H}^+]+\text{CH}_3\text{OH}+\text{H}_2\text{O}$).

3.1.1. Study on the stability of **F-oBOH** in aqueous solution

According to Franz's report [38], the fluorescein connecting to boronate ester cannot bind any metals strongly due to hydrolysis in aqueous solution. The hydrolysis of **F-oBOH** in the presence of Cu^{2+} in aqueous media at pH 7.0 was investigated by using fluorescence spectrophotometry. From Fig. 1a, the fluorescence intensity at 518 nm assigned to the opened-ring form of spirolactam based **F-oBOH** was dramatically increased upon the increment of time from 0 to 180 min. The ^1H NMR spectrum of **F-oBOH** in Fig. S2 showed the hydrolysis behavior in a similar fashion to that reported by Ma and coworkers [39]. The hydrazide part of **F-oBOH** was cleft to provide fluorescein and formylphenylboronic acid by Cu^{2+} in aqueous. This observation is indicative of the unstable **F-oBOH** in the presence of Cu^{2+} in aqueous solution.

3.1.2. Use of coordination nanoparticles to solve the problem

To overcome this limitation of the sensor in aqueous solution, we employed a new strategy for a fluorescence turn-on sensor system of **F-oBOH** using an adaptive self-assembly of subunits which could spontaneously form by flexibility following the shape of guest molecule in water. Kimizuka and coworkers [40–42] reported

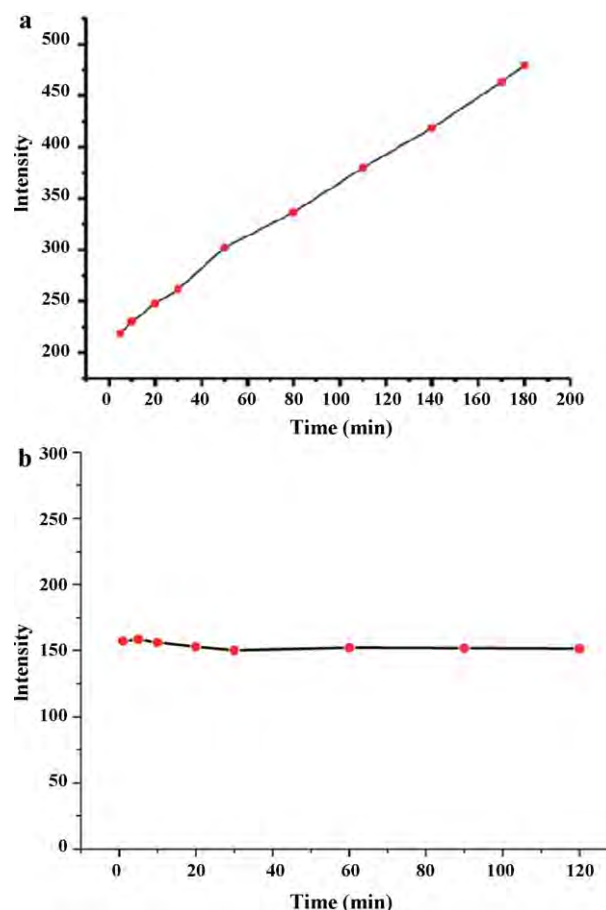
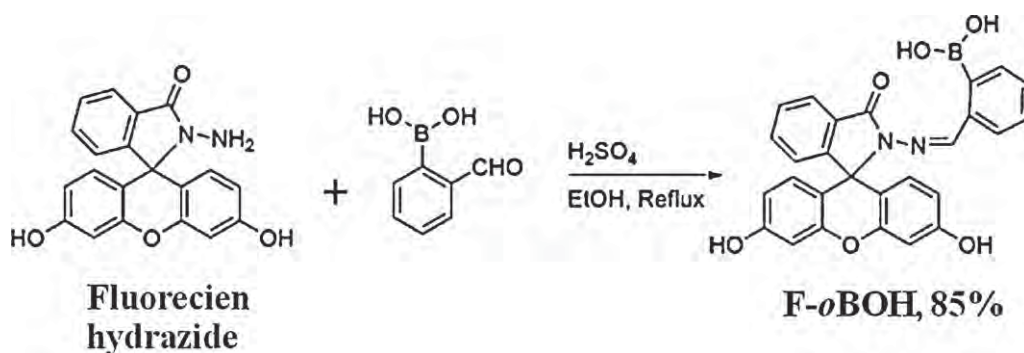


Fig. 1. The emission intensity at 518 nm of (a) **F-oBOH** and (b) **F-oBOH-AMP/Gd³⁺ CNPs** in the presence of copper(II) ion ($5 \mu\text{M}$) upon various time in the buffered solution pH 7.4 (5% EtOH:water) ($\lambda_{\text{ex}} = 498 \text{ nm}$).

that the coordination nanoparticles (CNPs) were naturally formed in water by the aids of several nucleotides, lanthanide ions, and dye molecules. In this research, the coordination nanoparticles were prepared by adding gadolinium(III) nitrate ($\text{Gd}(\text{NO}_3)_3$) into an aqueous mixture of adenosine monophosphate (AMP) and **F-oBOH** along with stirring. As shown in Fig. 2, the spherical nanoparticles (**F-oBOH-AMP/Gd³⁺ CNPs**) with average diameter of 60 nm were spontaneously formed and characterized by scanning and transmission electron microscopy.

We expected that the coordination nanoparticles would protect the hydrazone unit from hydrolysis due to the interior hydrophobic shell of the coordination nanoparticles. The fluorescence spectra at 515 nm of **F-oBOH** incorporated in AMP/Gd³⁺ CNPs in aqueous was



Scheme 1. Synthesis pathway of **F-oBOH**.

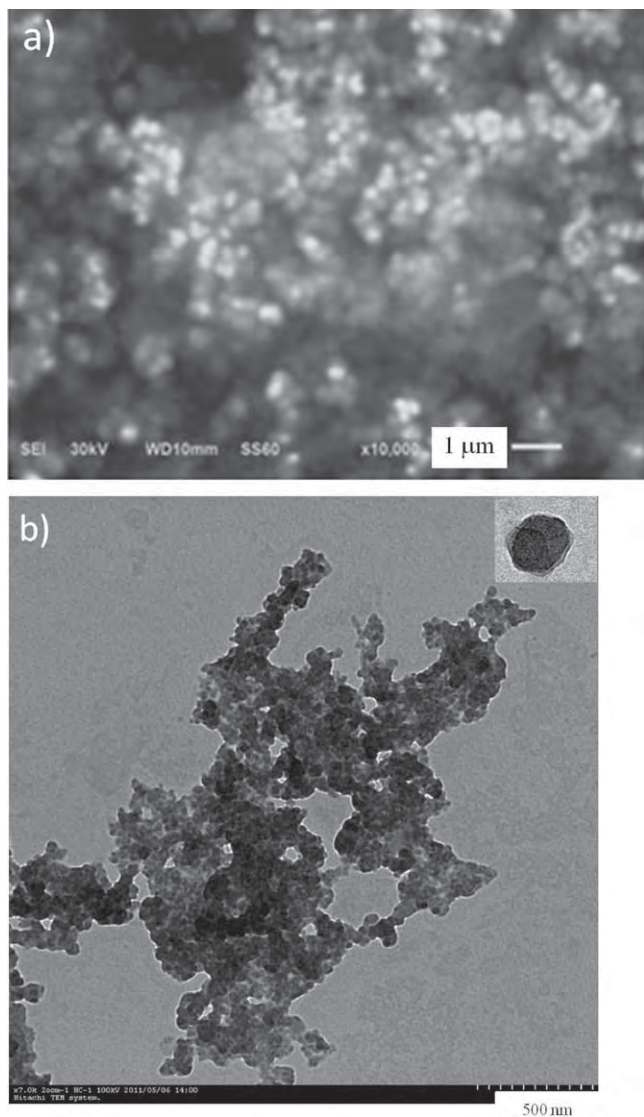


Fig. 2. (a) SEM image of **F-oBOH-AMP/Gd³⁺** CNPs and (b) TEM image of **F-oBOH-AMP/Gd³⁺** CNPs.

constant upon allowing to stand for 120 min as shown in Fig. 1b. This implied that **F-oBOH** incorporated in AMP/Gd³⁺ nanoparticles was very stable in aqueous solution. Therefore, the adaptive coordination nanoparticles network using AMP and Gd³⁺ could protect the hydrolysis of imine based **F-oBOH** by water.

3.1.3. Study on sensing ability of **F-oBOH-AMP/Gd³⁺** CNPs_{CN⁻} toward cations

In preliminary studies, the fluorescence spectra of **F-oBOH-AMP/Gd³⁺** CNPs_{CN⁻} was measured in buffered solution with respective metal cations including Cu²⁺, Co²⁺, Cd²⁺, Ni²⁺, Ag⁺, Hg²⁺, Mg²⁺ and Zn²⁺. The development of absorption band at 515 nm of the **F-oBOH-AMP/Gd³⁺** CNPs_{CN⁻} in the presence of Cu²⁺ is an indicative of the ring-opening form of spirolactam ring based **F-oBOH**. In the case of other transition metal ions, from Fig. 3, the fluorescence spectra of **F-oBOH** were changed insignificantly. This suggested that **F-oBOH-AMP/Gd³⁺** CNPs showed high selectivity toward Cu²⁺. Therefore, the nanoparticles network in the presence of Cu²⁺ can be used to detect anions using spectrofluorometry.

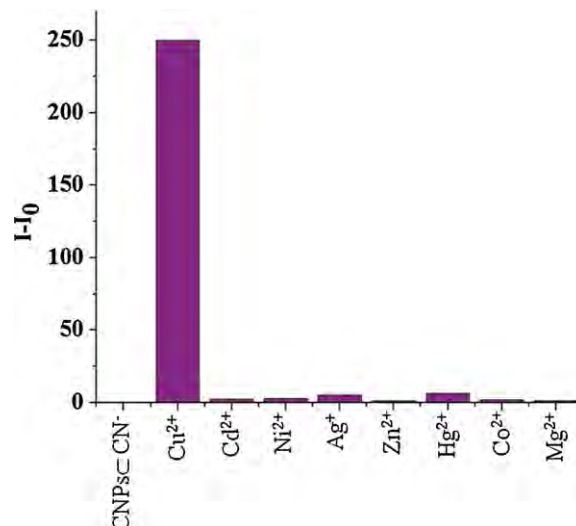


Fig. 3. Fluorescence intensity of **F-oBOH-AMP/Gd³⁺** CNPs_{CN⁻} at 515 nm in the presence of different cations (50 μM) in HEPES (0.1 M) pH 7.4 (λ_{ex} = 492 nm).

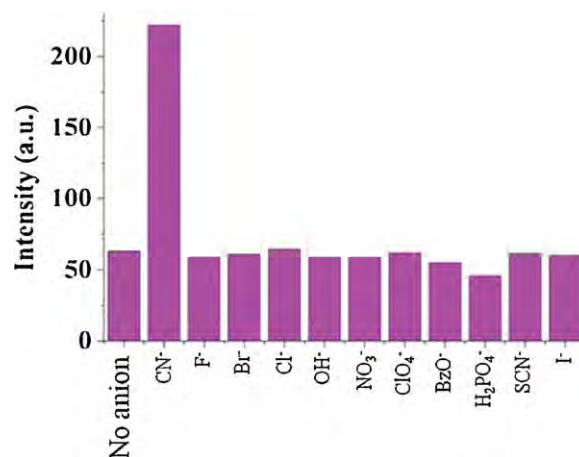


Fig. 4. Fluorescence intensity of **F-oBOH-AMP/Gd³⁺** CNPs_{Cu²⁺} at 515 nm in the presence of different anions (500 μM) in HEPES (0.1 M) pH 7.4 (λ_{ex} = 492 nm).

3.1.4. Study on sensing ability of **F-oBOH-AMP/Gd³⁺** CNPs_{Cu²⁺} toward anions

Such the boronic acid of **F-oBOH** has a vacancy site for anion substitution. Interactions of various anions toward the boron center of **F-oBOH-AMP/Gd³⁺** CNPs were evaluated by spectrofluorometry. As shown in Fig. 4, a fourfold of fluorescence enhancement of **F-oBOH-AMP/Gd³⁺** CNPs_{Cu²⁺} at 515 nm was particularly observed upon addition of CN⁻. These results revealed that **F-oBOH-AMP/Gd³⁺** CNPs_{Cu²⁺} possessed an excellent selectivity toward CN⁻. Presumably, CN⁻ bound boronic acid would induce the negative charge on boron center and subsequently promote the coordination of Cu²⁺ on the spirolactam unit resulting in the fluorescent enhancement.

Moreover, the naked-eyed sensing was performed in aqueous as shown in Fig. 5. The color changes from colorless to sky blue and the strong luminescence of **F-oBOH-AMP/Gd³⁺** CNPs_{Cu²⁺} upon binding with CN⁻ were remarkably observed. In comparison, **F-oBOH-AMP/Gd³⁺** CNPs_{Cu²⁺}, AMP/Gd³⁺ CNPs in the presence of Cu²⁺ and AMP/Gd³⁺ CNPs in the presence of Cu²⁺ and CN⁻ did not exhibit any change of optical properties. The results suggested that **F-oBOH** in the presence of Cu²⁺ acts as a cyanide sensor in

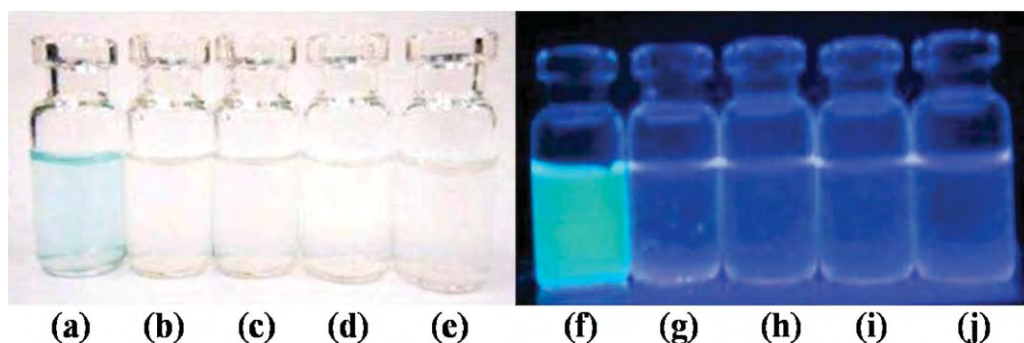


Fig. 5. Photograph of Naked-eyes of (a) **F-oBOH-AMP/Gd³⁺** CNPs in the presence of Cu^{2+} 45 equiv. and CN^- 90 equiv., (b) **F-oBOH-AMP/Gd³⁺** CNPs in the presence of Cu^{2+} 45 equiv., (c) **AMP/Gd³⁺** CNPs in the presence of Cu^{2+} 45 equiv. and CN^- 90 equiv., (d) **AMP/Gd³⁺** CNPs in the presence of Cu^{2+} 45 equiv. and luminescent of (e) **F-oBOH-AMP/Gd³⁺** CNPs in the presence of CN^- 45 equiv. and the samples were illuminated by the 356 nm UV light for (f) **F-oBOH-AMP/Gd³⁺** CNPs in the presence of Cu^{2+} 45 equiv. and CN^- 90 equiv. (g) **F-oBOH-AMP/Gd³⁺** CNPs in the presence of Cu^{2+} 45 equiv. (h) **AMP/Gd³⁺** CNPs in the presence of Cu^{2+} 45 equiv. and CN^- 90 equiv. (i) **AMP/Gd³⁺** CNPs in the presence of Cu^{2+} 45 equiv. (j) **F-oBOH-AMP/Gd³⁺** CNPs in the presence of CN^- 45 equiv.

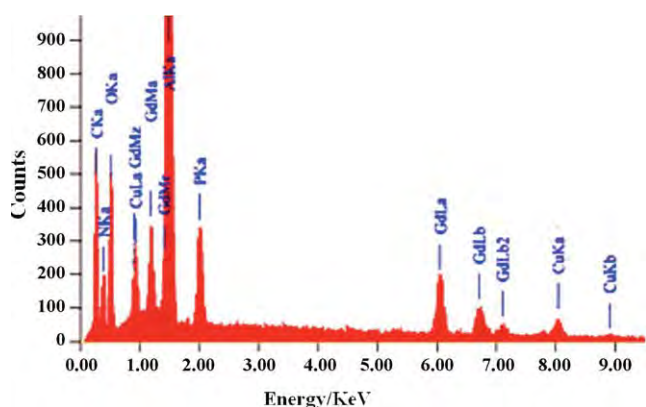


Fig. 6. Energy dispersive X-ray spectroscopy (EDS) spectrum of **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} CN^- (Al peak is from the Al stub).

aqueous solution giving a visual detection when it was encapsulated in **AMP/Gd³⁺** CNPs.

To verify the hypothesis for the structural complexation of **F-oBOH-AMP/Gd³⁺** CNPs, The stoichiometry of **F-oBOH-AMP/Gd³⁺** CNPs and Cu^{2+} and CN^- was analyzed by Job's plot method. The Job's plot analysis to investigate the stoichiometry of **F-oBOH** and Cu^{2+} or CN^- showed the complexation of **F-oBOH** incorporated in **AMP/Gd³⁺** CNPs and Cu^{2+} in a 1:1 binding mode and **F-oBOH** incorporated in **AMP/Gd³⁺** CNPs Cu^{2+} and CN^- also showed the 1:1 stoichiometry (as shown in Figs. S3 and S4).

The results suggested that one Cu^{2+} coordinated to spirolactam and one CN^- bound at a vacancy site of the boron center of **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} without the replacement of CN^- on both hydroxyl based boronic acid. Corresponding data from all Job's plot experiments and Kimizuka's reports [43,44], We proposed that Gd^{3+} could bind to the boron center and, thus, possibly function as a linkage on hydroxyl based boronic acid to form **F-oBOH-AMP/Gd³⁺** CNPs.

Additionally, we also characterized the incorporation of Cu^{2+} into **F-oBOH-AMP/Gd³⁺** CNPs using the energy dispersive X-ray spectroscopy (EDS). The **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} CN^- was prepared and washed with water several times in order to remove the remaining Cu^{2+} and CN^- in the solution. Then, the sample was brought to analyze the incorporated Cu^{2+} and other composition of coordination nanoparticles by EDX analysis.

From Fig. 6, the EDX spectrum shows the characteristic peak of copper element at 8.04 eV ($\text{Cu K}\alpha$), attributing to the incorporated Cu^{2+} in **F-oBOH-AMP/Gd³⁺** CNPs. The characteristic peaks

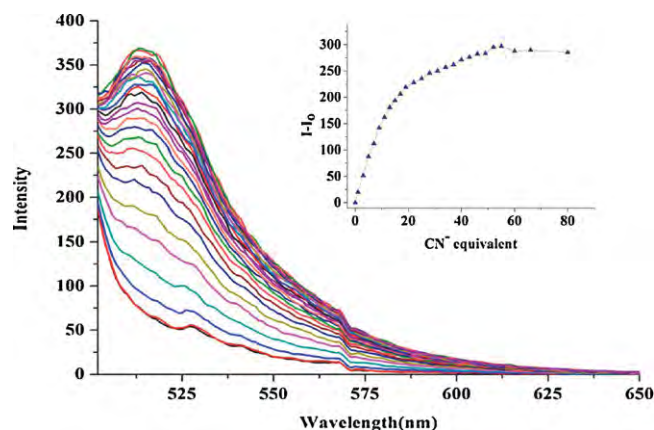


Fig. 7. Fluorescence titration spectra of **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} in HEPES (0.1 M) pH 7.4 upon addition of CN^- (0–80 equiv.) at emission band at 515 nm. Inset: Titration curve of **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} and CN^- (λ_{ex} , λ_{emis} = 492, 515 nm).

of gadolinium element at 6.05 eV ($\text{Gd K}\alpha$) and phosphorus at 2.01 eV ($\text{PK}\alpha$) assigned to the phosphorous element of adenosine monophosphate (AMP) were also observed. Thus, the EDX analyses give a strong evidence of the formation of Cu^{2+} incorporated into the **F-oBOH-AMP/Gd³⁺** CNPs.

The fluorescence-sensing ability of **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} toward CN^- was studied in HEPES pH 7.4. From Fig. 7, the fluorescence emission at 515 nm of the complexation increased as a function of cyanide concentration. The stability constant of **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} with CN^- calculated by Specfit 32 program in a 1:1 complexed fashion gives the log K values of 3.97 (as shown in Fig. S5).

The detection limit of **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} toward CN^- was examined by fluorescence spectroscopy in HEPES pH 7.4 solution providing 4.03 μM (0.198 ppm) which is nearly consistent with the minimum level defined by the World Health Organization [45].

The photograph of **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} upon adding the different concentration of the CN^- (20–400 μM) demonstrates the process of color changes of **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} from colorless to sky blue. According to Fig. 8, the visual detection limit of **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+} toward CN^- is approximately 20 μM (0.52 ppm).

3.1.5. Effect of interference for determination of CN^- by **F-oBOH-AMP/Gd³⁺** CNPs Cu^{2+}

The effect of foreign substances was investigated by considering a standard solution of CN^- to the added interference species.



Fig. 8. Naked-eyes of **F-oBOH**-AMP/Gd³⁺ CNPs/Cu²⁺ in HEPES (0.1 M) pH 7.4 in the presence of different concentrations of CN[−]. From left to right ($\times 10 \mu\text{M}$): **F-oBOH**-AMP/Gd³⁺, 0, 2.0, 4.0, 7.0, 10.0, 20.0, 30.0, and 40.0.

Table 2

Determination of CN[−] in drinking water sample.

Added CN [−] (μM)	CNPs/Cu ²⁺	
	Found (μM)	%Recovery
10	10.47	105
15	15.67	104
25	25.27	101
30	30.98	103
45	46.29	103

The tolerance amount and the value (%) of relative error of **F-oBOH**-AMP/Gd³⁺ CNPs/Cu²⁺ toward anions are collected in Table 1. The evaluated principle for interferences is fixed at a $\pm 10\%$ relative error. From Table 1, it was found that the coexisting substances showed slight effects on the determination of cyanide anion for **F-oBOH**-AMP/Gd³⁺ CNPs/Cu²⁺. In the case of H₂PO₄[−], the tolerance limit value cannot be determined due to the interruption of H₂PO₄[−] to the formation of the coordination nanoparticles [41]. Furthermore, H₂PO₄[−] caused a pH changes of the solution of **F-oBOH**-AMP/Gd³⁺ CNPs/Cu²⁺. This implied that **F-oBOH**-AMP/Gd³⁺ CNPs/Cu²⁺ can be applied to selectively sensing cyanide ion in the coexisting anions.

3.1.6. Use of **F-oBOH**-AMP/Gd³⁺ CNPs/Cu²⁺ for detecting CN[−] in drinking water

The nanoparticles network in the presence of Cu²⁺ was applied in the analysis of CN[−] in commercial drinking water by standard addition as listed in Table 2. Average % recoveries of the spike samples using **F-oBOH**-AMP/Gd³⁺ CNPs/Cu²⁺ were 103.2. This proposed method, therefore, was a good promising way for CN[−] detection.

4. Conclusions

We have shown that **F-oBOH** could be encapsulated in the adaptive nucleotide/lanthanide coordination nanoparticles, AMP/Gd³⁺ CNPs, to avoid hydrolysis upon using in water. Interestingly, this methodology gave a promising selectivity to detect CN[−] in the presence of Cu²⁺ in HEPES pH 7.4 with detection limits obtained from spectrofluorometry and the visual change of 4.03 μM (0.198 ppm) and 20 μM (0.52 ppm), respectively. Furthermore, **F-oBOH**-AMP/Gd³⁺ CNPs/Cu²⁺ can be used for cyanide sensing without separation of any interference anions even with hydroxide anion. We have demonstrated that this proposed method can be used to detect CN[−] in drinking water with high accuracy. Finally, this study would be beneficial in further development of spectroscopic methods for the probe bearing the cleavable active bonds in water.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.12.024.

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Discrimination of nucleotides by single fluorescence sensor under solvent-dependent recognition patterns

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ABSTRACT

The chemosensor based on Zn complex of 1-[bis(pyridine-2-ylmethyl)amino]methylpyrene (**1-Zn**) was synthesized for detection of nucleotides in aqueous solution. This sensor has a high selectivity for PPI in aqueous solution. However, in the solution of DMSO:HEPES (0.01 M, pH 7.4, 90:10, v/v), **1-Zn** selectively bound nucleoside polyphosphates such as ATP and UTP with a large fluorescence enhancement. Tremendously different recognition patterns of **1-Zn** toward various phosphate anion based nucleotides in the two solvent systems encouraged us to discriminate the structurally similar nucleotide anions. The principal component analysis (PCA) was applied to discriminate 10 nucleotides in two solvent systems. This approach permits the identification of 10 phosphate analytes with nearly 100% accuracy from a single sensor in two solvent systems. To illustrate the utility of this approach for ATP hydrolysis application, the ratios between the product of reaction (PPI) and the reactant (ATP) were plotted versus the ratios of fluorescence intensity. The graph can be used for quantitative prediction of the PPI and ATP ratios with the detection limit of 2:8.

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1. Introduction

Detection of specific biological molecules by artificial chemosensors by fluorescence spectroscopy, a versatile technique with high sensitivity, rapid response, and easy performance, is currently of interest subject to pursue [1]. The fluorescent probe for biological anions is actively investigated since these anions play several important roles in biological processes. Additionally, anions also involved in many enzymatic processes and signal transduction in cellular communications. For example, ATP is a universal energy source for various cellular functions in all living systems and also incorporated into the process of DNA replication and transcription [2,3]. In addition to these fundamental intracellular roles, nucleoside polyphosphates such as ATP, ADP, and a variety of adenosine polyphosphates also play pivotal extracellular roles as signal transducers [4]. In particular, pyrophosphate is the product of ATP hydrolysis under cellular conditions.

Interestingly, many receptors have been employed to capture and detect anions in organic solvents because the solvation

energy is relatively small and the electrostatic interactions operate effectively. However, most biological applications are relevant in aqueous media. Artificial receptors in aqueous systems showed poor affinity for recognition of anions due to the influence of the strong hydration [5].

An alternative approach for efficient binding of PPI in aqueous media stemmed from metal coordination interactions. In devising a chemosensing ensemble, transition metal ions, such as Zn²⁺ and Cu²⁺ have commonly been incorporated into ligands due to their abilities to provide beneficial electrostatic interactions in polar solvents such as water [6]. It is well known that dipicolylamine (Dpa) can bind with Zn²⁺ using lone pair electrons of nitrogen atom [7,8]. Zinc(II)-dipicolylamine complexes have been widely used as binding units for phosphate chemosensors [9–12]. However, the strategy for discrimination between structurally similar analytes of nucleotide anions using only one sensor is still challenging. Thus, a trial that has received attention is principle component analysis (PCA) method. Typically, PCA is most widely used for statistical evaluation of the array responses, not for any single sensor [13–18]. Suslick and co-workers [19] reported a colorimetric sensor array of nanoporous pigments that is capable of differentiating various mono and disaccharides including sweeteners. Anzenbacher and co-workers [20] illustrated eight-sensor arrays that discriminated 10 inorganic anions and then used the fluoride content for identification of several toothpaste brands. Furthermore, PCA

Abbreviations: Dpa, dipicolylamine; PCA, principal component analysis.

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method is also used to describe a certain level of discrimination between similar structures of nucleotide anions. Anslyn and co-workers [21] demonstrated an excellent combinatorial library of sensors and indicator displacement assay to discriminate between ATP, GTP and AMP in aqueous solutions. Nevertheless, a few report [22–24] involved the simple experiments using a single sensor with PCA method. Hewage and Anslyn demonstrated a single receptor, squaraine, acting as a host and an indicator to differentiate five metals and a series of thiols [25].

From the previous researches, our challenge is to search for the easy and uncomplicated PCA method to discriminate the structurally similar analytes of 10 nucleotide anions in different conditions of media by using a single sensory molecule. The attractive fluorescence sensor based on pyrene and Dpa-Zn complex provides a high selectivity for PPi in aqueous solution while this sensor in DMSO:H₂O (90/10) coordinates preferentially with UTP and ATP. Since this sensory molecule can give different recognition patterns in different solvent systems, all subsequent fluorescent spectra were thus combined to create a PCA plot to separate the clusters of nucleotides. In addition, we have applied the PCA method for further quantitative determination of the ratios of PPi and ATP for ATP hydrolysis in a biomimetic application.

2. Experimental

2.1. General

Nuclear magnetic resonance (NMR) spectra were recorded on Varian Mercury 400 MHz and Bruker 500 MHz nuclear resonance spectrometer (in CDCl₃, DMSO-*d*₆ and D₂O). ESI HRMS were recorded on micrOTOF-Q II BRUKER. Fluorescence spectra were performed on Varian Cary Eclipse spectrofluorometer. The light source is a pulsed xenon lamp and a detector is a photomultiplier tube.

Reagents for synthesis were used without further purification. Sodium triacetoxyborohydride, zinc perchlorate hexahydrate and nucleotide anion (ATP) were obtained from Aldrich. Dipicolylamine and pyrenecarboxaldehyde were obtained from TCI. HEPES: 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) CMP, GMP, GDP, AMP, UTP, UDP and UMP were obtained from Sigma. ADP was obtained from Calbiochem.

Dimethylsulfoxide was reagent grade purchased from Lab scan. Commercial grade solvents such as dichloromethane and ethyl acetate were purified by distillation before used. Dichloromethane for set up the reaction were dried over calcium hydride and freshly distilled under nitrogen atmosphere prior to use. Column chromatography was carried out on silica gel (Kieselgel 60, 0.063–0.200 mm, Merk). Thin layer chromatography (TLC) was performed on silica gel plates (Kieselgel 60, F₂₅₄, 1 mm). Compounds on TLC plates were detected by the UV-light.

2.2. Synthesis of **1-Zn**

A solution of Zn(ClO₄)₂ in 5 mL MeOH (0.171 g, 0.47 mmol) was added dropwise into a solution of **1** [26] (0.112 g, 0.27 mmol) in 5 mL MeOH. After stirring overnight at room temperature, the corresponding precipitate was filtered and washed with MeOH to give **1-Zn** (0.21 g, 100%) as a light yellow powder. ¹H NMR (DMSO-*d*₆) δ 8.71 (s, 2H), 8.42 (s, 1H), 8.37–8.13 (m, 9H), 7.94 (s, 1H), 7.68 (s, 2H), 7.56 (d, *J*=6.4 Hz, 2H), 4.55 (s, 2H), 4.35 (d, *J*=15.2 Hz, 2H), 3.69 (d, *J*=15.6 Hz, 2H). ESI HRMS: calcd for C₂₉H₂₃N₃Cl₃O₁₂Zn *m/z* [M+(ClO₄)₃][−] 773.9639, found 773.9829.

2.3. Computational method

The structure optimizations of PPi/(**1-Zn**)₂, ATP/(**1-Zn**)₂ complexes and their components were carried out using density functional theory (DFT) method. The DFT computations have been performed with hybrid density functional B3LYP which is the Becke's three-parameter exchange functional [27] with the Lee–Yang–Parr correlation functional [28] combined with the Los Alamos LanL2DZ split-valence basis set called as B3LYP/LANL2DZ level of theory. All computations were performed using GAUSSIAN 03 program.

2.4. Complexation studies by fluorescence spectrophotometry

Typically, a solution of **1-Zn** (1 × 10^{−5} M) in both two solvent systems (90:10 and 1:99, v/v of DMSO:HEPES (0.01 M, pH 7.4)) was prepared. All of nucleotide anions (ATP, ADP, AMP, UTP, UDP, UMP, GDP, GMP, CMP and PPi) were prepared at the concentration of 1 × 10^{−3} M in HEPES buffer 0.01 M, pH 7.4. Fluorescent spectra of **1-Zn** and anion complexes were recorded from 356 to 600 nm at ambient temperature. For fluorescence titration, the solution of anion was added directly to 2.00 mL of **1-Zn** (1 × 10^{−5} M) in a 1-cm quartz cuvette by micropipette and the portion of the mixture solution was stirred for 5 min. The fluorescent spectra were measured after each addition until the system reached the equilibrium point.

2.5. Job's plot between **1-Zn** and PPi anions by fluorescence spectrophotometry

Typically, a solution of **1-Zn** (1 × 10^{−5} M) in the HEPES buffer were prepared by adding 250 μL of a stock solution of **1-Zn** (1 × 10^{−3} M in DMSO) in a 25 mL volumetric flask. The solution of PPi (1 × 10^{−5} M) was diluted from a stock solution of PPi (1 × 10^{−3} M in HEPES buffer). A portion of **1-Zn** and PPi in the range of mole ratio from 1:0 to 0:1 was putted in a 1-cm quartz cuvette and stirred for 5 min prior to measurement of fluorescence spectra.

2.6. Principle component analysis (PCA) method

For PCA analysis, the solution of 200 μL analytes (1 × 10^{−3} M in HEPES buffer 0.1 M, pH 7.4) was added directly to 2.00 mL of **1-Zn** (1 × 10^{−5} M) in a 1-cm quartz cuvette by micropipette and the mixture was stirred for 5 min. The fluorescent spectra were measured after each addition. The sample was measured at 5 replicates in each analytes. PCA calculation was evaluated by the responsive fluorescence spectra from 356 to 600 nm using MATLAB 7.11 (version R2011a).

2.7. NMR studies

A solution of a **1-Zn** (8.37 mg, 1.74 × 10^{−5} mol, 4.35 × 10^{−2} M), a solution of of a **1-Zn** (8.37 mg, 1.74 × 10^{−5} mol, 4.35 × 10^{−2} M) containing one equivalent of ATP (1.74 × 10^{−5} mol) and a solution of ATP in DMSO-*d*₆:D₂O (7:1, v/v, 0.4 mL) were prepared in NMR tubes. All of samples were measured in ¹H NMR spectroscopy and the complexed solution of **1-Zn** and ATP in DMSO-*d*₆:D₂O was measured in 2D-NOESY spectroscopy.

3. Results and discussion

The synthesis of 1-[bis(pyridine-2-ylmethyl)amino]-methylpyrene **1** was carried out according to the reported procedure [26]. The **1-Zn** complex was prepared by mixing **1** and one equivalent of zinc perchlorate in methanol with 100% yield, as shown in Scheme 1. The ¹H NMR spectrum of **1-Zn**

Scheme 1. Synthesis of sensor **1-Zn**.

showed the signal of aromatic pyridine protons (H_1 – H_4) at 8.71, 7.94, 7.68, 7.56 and methylene protons (H_5 – H_9) at 4.55, 4.35, 3.69 ppm (Fig. S2 in Supporting Information). Moreover, the HRMS of this complex exhibits the intense peak at 773.9829 m/z which agrees with the structure of **1-Zn** complex (Fig. S4 in Supporting Information).

Compound **1** containing three nitrogen atom of dipicolylamine moiety is favorable to chelate Zn^{2+} ion. This sensor has a pyrene unit as a fluorophore. Thus, the sensor bound to various nucleotides by using electrostatic interactions based on metal–ligand coordination. The sensing ability of **1-Zn** with nucleotides was investigated by fluorescence spectrophotometry in aqueous solution with different systems. Fig. 1a exhibits the effect of anions on the fluorescence spectra of sensor **1-Zn** in an aqueous solution of DMSO:HEPES buffer (10 mM, pH 7.4 with 1:99, v/v). The characteristic emission band of sensor **1-Zn** appeared at 360–450 nm which was assigned to the pyrene monomer emission. Upon the addition of various nucleotide anions, PPI showed an obvious fluorescence enhancement of monomer emission. A slight fluorescence enhancement was observed upon addition of ATP and ADP, while other nucleotides show slight change in their spectra. These results suggested that sensor **1-Zn** has a higher sensitivity for PPI over other nucleotides in the order of $PPI > ATP > ADP$.

From the fluorescent titration, the log K value of **1-Zn** complexed with PPI obtained by SPECFIT32 program using the 1:2 binding mode of **1-Zn** and PPI was 10.22 ± 0.03 (Fig. 2). In the case of ATP and ADP, their log K values cannot be determined possibly caused by the simultaneous formation of 1:1 and 2:1 ratios for host:guest binding mode. [29] The Job's plot analysis for the binding mode of **1-Zn** and PPI suggests a 2:1 stoichiometry (inset Fig. 2). The selectivity of **1-Zn** for PPI over ATP can be explained by the basis of the charge density of phosphate unit in molecule. The total negative charge

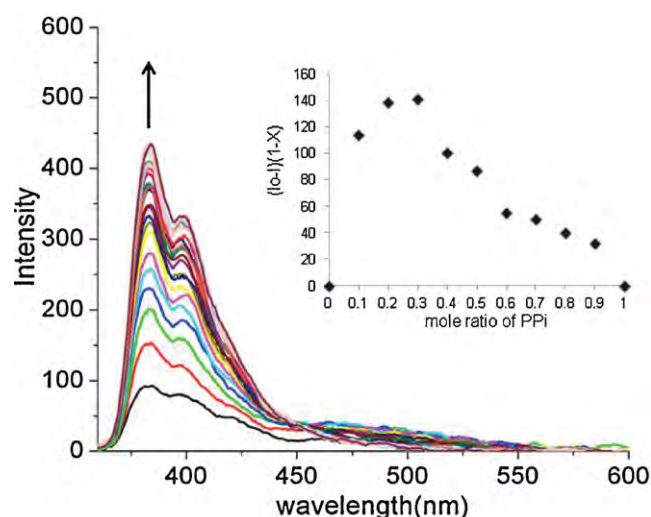


Fig. 2. Fluorescence titration of **1-Zn** (1×10^{-5} M) upon addition of PPI in DMSO–HEPES buffer (0.01 M, pH 7.4) (1:99, v/v). (Inset) Job's plot of **1-Zn** with PPI.

density of phosphate unit based PPI is much higher than that of phosphate unit based ATP [30,31]. Consequently, the positive Zn^{2+} complex is favorable to bind PPI over ATP.

Furthermore, to develop the PPI-sensing application of **1-Zn** on solid support, the word “PPI” was prepared by the solution of **1-Zn** on the filter paper using paintbrush and then dipped in PPI solution. Upon exposure to the UV irradiation at 365 nm, the word “PPI” exhibited a tremendous brighter luminescence as shown in Fig. 3b.

Actually, most of anion sensors function in organic solvent because the solvation energy is relatively small and the electrostatic interactions operate effectively. Hence, we also investigated the binding properties of **1-Zn** and nucleotides in organic solvent (90% of DMSO in H_2O). Fig. 1b shows the fluorescence changes upon various analytes such as UTP, ATP, UDP, ADP, GDP, UMP, AMP, GMP, CMP and PPI in DMSO:HEPES (0.01 M, pH 7.4, 90:10, v/v). Upon the addition of nucleoside triphosphate such as UTP and ATP, the observation of the strong fluorescence enhancement of **1-Zn** at 383 nm indicated a high affinity toward UTP and ATP. For nucleoside diphosphates (ADP, UDP, GDP), the emission band was slightly changed, while nucleoside monophosphate (AMP, GMP, CMP, UMP) did not induce any fluorescence changes of **1-Zn**. In contrast, PPI

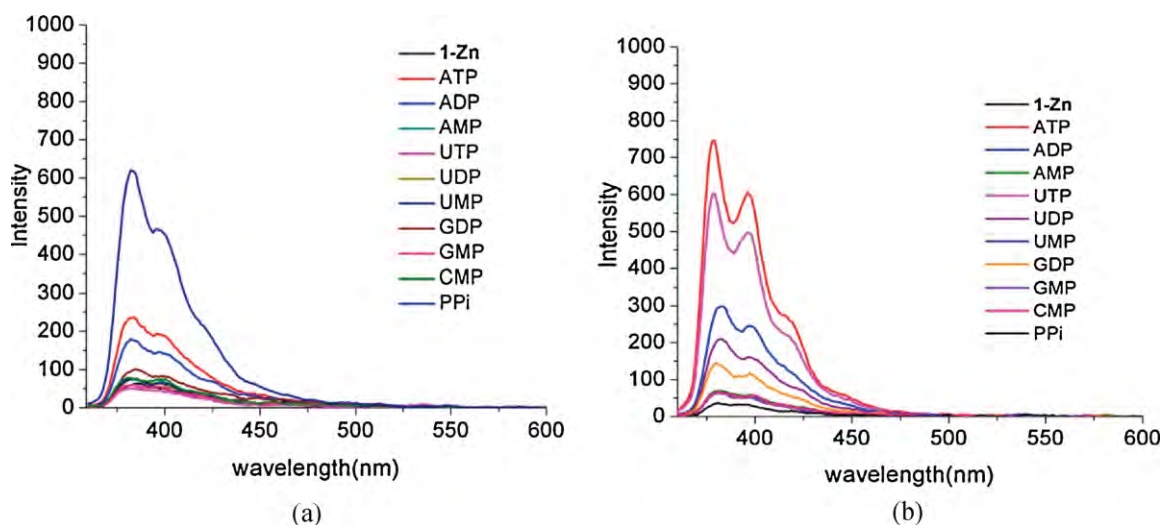


Fig. 1. Fluorescence emission spectra of **1-Zn** (1×10^{-5} M) in the presence of various nucleotides (10 equiv) in DMSO–HEPES buffer (0.01 M, pH 7.4) (a) 1:99 (v/v) (b) 90:10 (v/v). Excitation wavelength 346 nm.



Fig. 3. Image patterns of **1-Zn** dip with PPI solution on a filter paper (a) in the visible light and (b) the formation of patterned fluorescence image of word “PPI” under UV irradiation at 365 nm.

Table 1

The association energies of overall associations of ATP complexed with **1-Zn** and PPI complexed with **1-Zn**, in gas phase and aqueous phase, and free energy of their association in aqueous solution.

Overall association	ΔE^a		ΔG^{298a}
	In vacuo ^b	In aqueous ^c	In aqueous ^c
2 1-Zn + ATP $\rightarrow$ ATP/(1-Zn) ₂	−922.16	−105.78	−90.73
2 1-Zn + PPI $\rightarrow$ PPI/(1-Zn) ₂	−1100.64	−115.82	−106.80

^a In kcal/mol.

^b Computed using the B3LYP/LANL2DZ method.

^c Computed using the CPCM(UAKS)/B3LYP/LANL2DZ method.

induced a small fluorescence quenching at 383 nm. The **1-Zn** would, thus, preferably form complexes with the polyanionic species over the monoionic phosphate [32].

The structure optimizations of PPI/(**1-Zn**)₂, ATP/(**1-Zn**)₂ complexes and their components were carried out using DFT at the B3LYP/LANL2DZ level of theory. All computations were performed using GAUSSIAN 03 program [33]. The optimized structures of PPI and ATP complexes were illustrated in Fig. 4 [34–36]. Upon complexation of **1-Zn**-PPI and **1-Zn**-ATP, the strong ionic bonds between cationic Zn²⁺ ion and oxygen atom of PPI and ATP occurred. The interactions of PPI/(**1-Zn**)₂ and ATP/(**1-Zn**)₂ complexes mainly stemmed from six and five hydrogen bonding interactions, respectively. Furthermore, the most stable form of PPI/(**1-Zn**)₂ and ATP/(**1-Zn**)₂ complexes cannot induce the excimer formation of pyrene moieties. This result is consistent with those from the emission spectra.

The association energies of overall associations of **1-Zn** complexed with ATP or PPI in the gas phase and aqueous phase, and free energy of their association in aqueous solution are collected in Table 1. In aqueous media, the association energies and the Gibbs’ free energies were derived from the CPCM method at the B3LYP/LANL2DZ level. The results showed that the formation of PPI/(**1-Zn**)₂ was more stable than that of the ATP/(**1-Zn**)₂, either in gas phase or aqueous phase. Therefore, the PPI/(**1-Zn**)₂ association is a more thermodynamically favorable pathway in aqueous solution.

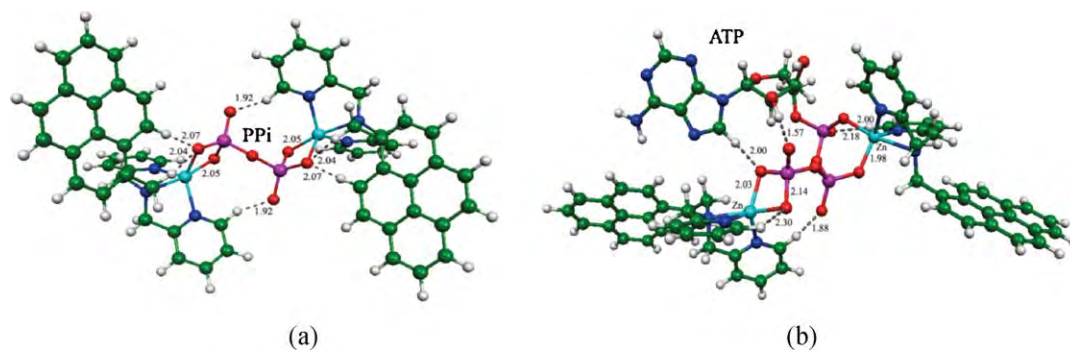


Fig. 4. The B3LYP/LANL2DZ optimized structures of (a) PPI/(**1-Zn**)₂ and (b) ATP/(**1-Zn**)₂ complexes. The bond lengths and bond distances are in Å.

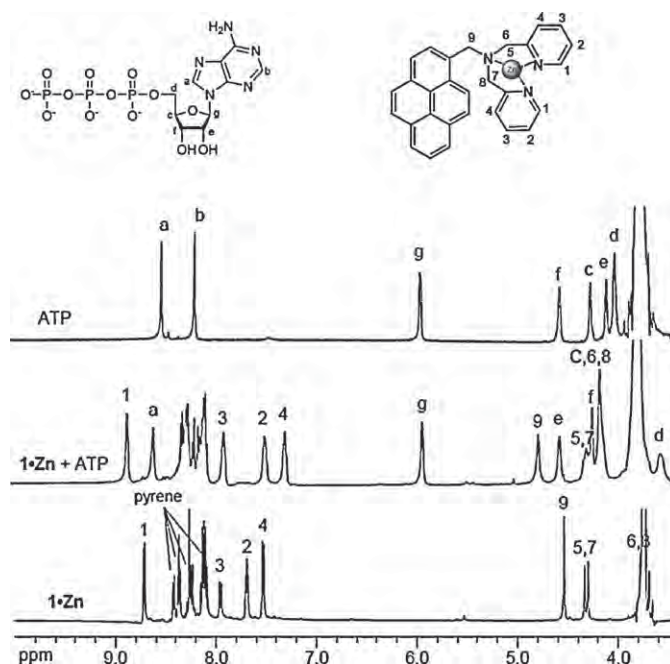


Fig. 5. Partial ¹H NMR spectra of **1-Zn**, **1-Zn** + ATP (1 equiv) and ATP in DMSO-*d*₆:D₂O (7:1, v/v) (500 MHz).

In order to gain the support for the binding mode between **1-Zn** and ATP, ¹H NMR experiment and 2D NOESY of **1-Zn** with ATP in the mix solvent of DMSO-*d*₆ and D₂O (7:1, v/v) were examined. ¹H NMR spectra of **1-Zn** in the absence and presence of ATP were shown in Fig. 5. Addition of one equivalent of ATP to the solution of **1-Zn** in DMSO-*d*₆ caused downfield shifts of aromatic protons (H₁) of pyridine moieties around 0.12 ppm. In addition, a slightly downfield shift of imidazole proton (H_a) of adenine based ATP at 8.48 ppm was observed. It was indicative of the strong hydrogen bonding interactions between these corresponding protons and the phosphate groups of ATP. On the contrary, the remaining pyridine protons in Dpa part of **1-Zn** including H₂ and H₄ underwent a large upfield

shift, whereas H_3 proton showed a small-upfield shift by 0.22, 0.26 and 0.068 ppm, respectively. The shifts of these protons revealed the geometry change due to ATP complexes [37]. Moreover, the significant shifts of CH_2 protons (H_5 , H_6 , H_7 , H_8 , H_9) demonstrated the perturbation of the aliphatic protons in Dpa part upon the complexation of ATP and **1-Zn**. To gain more important information about the structure of the complex, 2D NMR NOESY was also investigated. From the NOESY spectrum of **1-Zn** with ATP, the correlation peaks between adenosine protons based ATP and pyrene based **1-Zn** have not been observed. The result suggested that the binding mode stemmed from the interactions of Zn^{2+} ion with the phosphate part of ATP without the influence of the adenosine part (Fig. S5 in Supporting Information). The NOSEY data were consistent with the results from spectrophotometric and computation studies.

Considering two solvent systems, DMSO:HEPES (90:10, v/v) and aqueous solution, the binding affinity of **1-Zn** and nucleotides showed the absolutely different fluorescence intensity changes indicating the different recognition patterns. These results encouraged us to verify the discrimination of binding affinity between **1-Zn** and nucleotides by examining the fluorescence responses of complexes obtained from the two solvent systems. PCA is used to demonstrate a combinatorial library of a single sensor in the two solvent systems. This approach allows the discrimination of 10 nucleotide anions.

Principal component analysis (PCA) is a non-supervised approach utilized to reduce the dimensionality of the data set into a new coordinate system called “Principal Components (PCs)”. For each of five trials, the intensity value was recorded for each of 10 nucleotides. To identify patterns in the data, a principal component (PC) axis is calculated to lie in the direction of maximum variance in the original data. The first component (PC1) always represents the greatest variance and the second greatest variance is projected to the second component (PC2) and so on. In its simplest form, the transformation of the original data (X) using PCA can be expressed by

$$X = T \cdot P + E$$

where T is the score and P is the loading matrix. Only the scores are used in this paper to reveal clustering based on the similarity of analytes [38–42]. All data analysis using PCA with non-linear iterative partials least squares (NIPALS) method [38] were performed in MATLAB version 7.11 (R2011a).

The PCA results for **1-Zn** with various nucleotide anions in DMSO:HEPES (1:99, v/v) shows 99.6% variance along PC1, while the vertical PC2 axis displays 0.1% variance (Fig. S6 in Supporting Information). It can classify only the cluster of PPI from other nucleotides. To quantify the classification performance, linear discriminant analysis (LDA) with leave-one-out cross validation approach [38] was used to express the discrimination of the nucleotides. Cross-validated LDA shows 82% accurate classification for all 10 nucleotides. On the contrary, PCA score plot of 90% DMSO in HEPES buffer solution showed the horizontal PC1 axis with 99.1% of variance and illustrated 0.7% of variance along PC2 (Fig. S7 in Supporting Information). This PCA plot exhibited a different discrimination pattern from the mentioned system. In particular, the nucleoside triphosphates including ATP and UTP can be separated from the remaining nucleotides and LDA cross validation showed 74% correct classification in 50 cases. To improve a certain level of discrimination between 10 nucleotides, the recognition patterns obtained by fluorescent responses in the two solvent systems were combined and subsequently applied to PCA plots. A two dimensional score plot for the first two principal components (PC1 and PC2) representing nearly 100% of variance (as shown in Fig. 6) exhibits an impressive discrimination with clustering of the 10 nucleotides in the two solvent systems. The PCA score plot for this

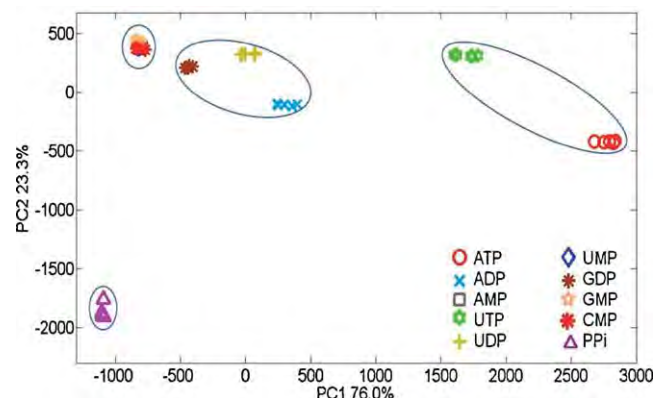


Fig. 6. PCA score plot of two PCs describing ca. 100% of the total variance for all 10 nucleotides (5 trials each) of **1-Zn** in mix between in DMSO:HEPES (1:99, v/v) and DMSO:HEPES (90:10, v/v).

combinatorial data showed 76.0% of the variance along the PC1 and the PC2 axis displayed 23.0% of the variance. Clearly, the PCA plot is capable to classify the representative number of phosphate anions such as inorganic pyrophosphate (PPI), triphosphate anions (UTP and ATP), diphosphate anions (ADP, UDP, and GDP), and monophosphate anion (UMP, AMP, GMP and CMP). Deeply considering, the appearance of ATP cluster on the right can be separated from UTP cluster in the right upper of the PCA score plot. The case of ADP, GDP and UDP clusters appeared in the left upper close to monophosphate group, while the PPI cluster appeared in the left lower corner. This pattern shows a large differentiation between the analytes [43].

Additionally, cross-validated LDA for class prediction illustrated a perfect discrimination of PPI, tri- and di- phosphate anions in 100% accuracy, with the exception of monophosphate anions (70% accuracy). However, cross-validated LDA showed 88% accuracy of overall prediction for 10 nucleotides which was a poor value. This may be caused by the unsuitable binding of **1-Zn** toward the monophosphate.

Interestingly, the PCA analysis can significantly separate between ATP, AMP and PPI. After a successful discrimination of ATP, AMP and PPI, we expected to extend the scope of our system to follow the amount of the PPI and ATP in the ATP hydrolysis which actually produces the AMP and PPI in the cellular system. In our hypothesis, the quantitative identification of the ratios of PPI and ATP using **1-Zn** in both solvent systems were achieved by the different fluorescent responses illustrated by PCA score values.

As anticipated, each pattern of the PCA score plot generated by the sensor with the ratio between PPI and ATP in the range of 0:10 to 10:0 is reduced to a few score and plotted in the new space (PC space) as shown in Fig. 7. The different recognition patterns of **1-Zn** toward various nucleotides can be detectable upon the addition of 10 equivalents of analyte. This is a rationalization that the ATP hydrolysis studies were measured in the range of 0:10 to 10:0 for PPI and ATP ratios (the spectral changes with various ratios of PPI and ATP shown in Fig. S8–10). The PCA score plot of a single sensor in aqueous system shows the linear relationship of PC1 and PC2 axes while that of the sensor in DMSO solution showed non-linearity. The PCA method shows the high cross reactivity to discriminate the ratio of PPI/ATP in particular of the aqueous system.

In our effort to evaluate the fluorescence responses from both solvent systems, the plot between the ratios of fluorescence intensity of the sensor in HEPES system and in DMSO (I_H/I_D) against the ratio of PPI and ATP (in the range of 0:10 to 10:0) was constructed. It exhibited the calibration curve with R^2 of 0.9848 as shown in Fig. 8. As expected, this result is possibly capable to be used for

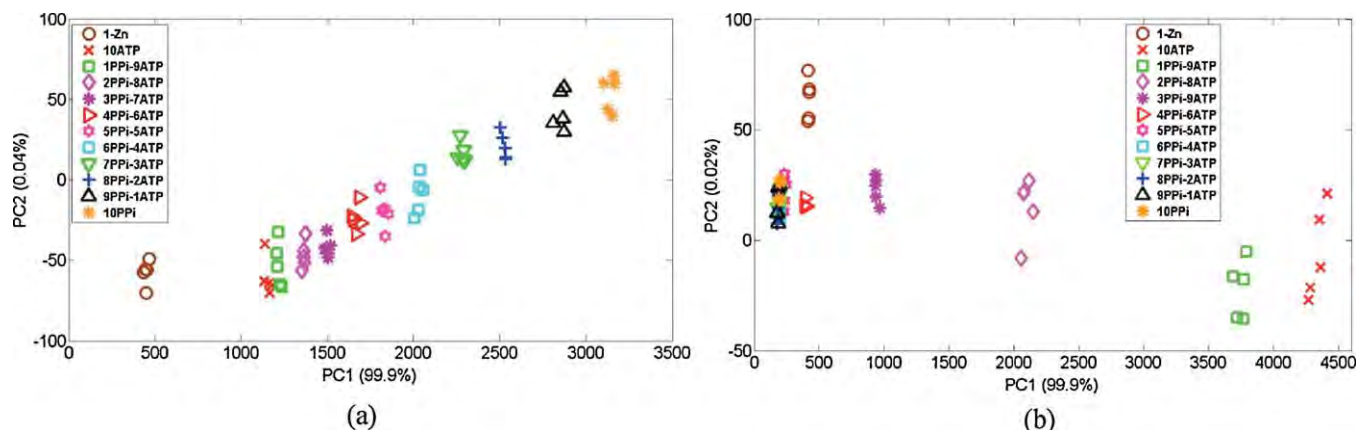


Fig. 7. PCA score plot of the ratio variation between PPI and ATP PCs describing ca. 100% of the total variance. PCA score plot shows clustering for all 11 samples (5 trials each) in (a) DMSO:HEPES (1:99, v/v) (b) DMSO:HEPES (90:10, v/v).

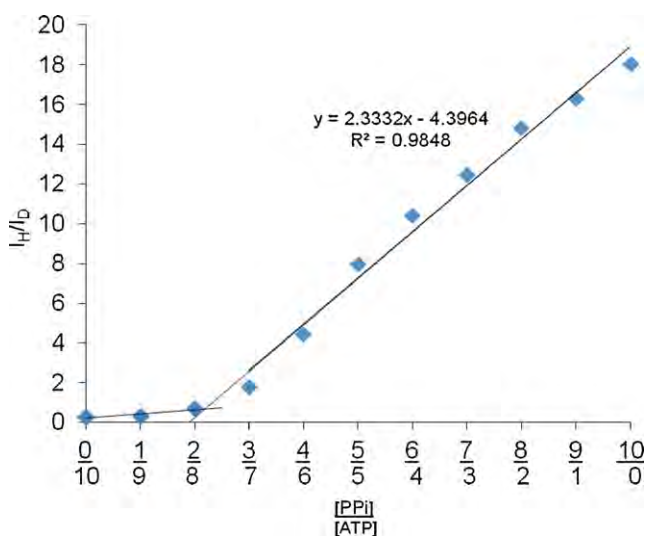


Fig. 8. Plot between mole ratio of PPI/ATP against the ratios of fluorescence intensity of the sensor in HEPES system and in DMSO.

quantitative prediction of the PPI and ATP ratios with the detection limit of 2:8 ratios.

4. Conclusion

The fluorogenic complex **1-Zn** based on pyrene dipicolylamine have been successfully synthesized. We have demonstrated that the design of optical signal transduction pyrene bearing dpa- Zn^{2+} center exhibits an excellent fluorescent chemosensor for PPI over other anions in an aqueous solution. The log K values of **1-Zn** complexed to PPI is 10.22. On the contrary, in DMSO, the sensor is more favorable to UTP, ATP than PPI. The complexation structure of **1-Zn** and ATP showed the promising binding mode relied on anion–cation recognition of phosphate unit and Zn^{2+} without the interactions between the adenosine based ATP and the pyrene moiety. We have successfully reported the different recognition pattern of **1-Zn** toward nucleotides and applied the beneficial PCA method by the combinatorial data of fluorescent intensity obtained from a single sensor in different solvent systems to discriminate the analytes. In biomimetic applications for ATP hydrolysis, the use of the combination of fluorescent responsive data from both solvent systems was expected to predict the ratio between PPI and ATP. We currently attempt to raise the impact of this systematic procedure

for sensing application of a variety of analytes in real-time analysis in the medical diagnosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.snb.2012.06.011>.

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บทสารพน # 4

เซ็นเซอร์โมเลกุลชนิดใหม่ที่ประกอบด้วยกรดโบโรนิก/ฟลูออเรสซิน สำหรับตรวจจับคอปเปอร์และไซยาไนด์ไอออน

NEW BORONIC ACID/FLUORESC EIN SENSORS FOR DETECTION OF COPPER AND CYANIDE IONS

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บทคัดย่อ

งานวิจัยนี้มีจุดมุ่งหมายที่จะสังเคราะห์และพิสูจน์เอกลักษณ์เซ็นเซอร์ตัวใหม่ที่มีอนุพันธ์ของฟลูออเรสซินและกรดโบโรนิก **F-mBOH** เพื่อใช้ตรวจวัดคอปเปอร์และไซยาไนด์ไอออน จากนั้นได้นำเซ็นเซอร์ **F-mBOH** มาศึกษาการเกิดสารประกอบเชิงซ้อนกับแคตไอออนชนิดต่าง ๆ ซึ่งใช้อะซิโตนไนไตรเป็นตัวทำละลาย โดยใช้เทคนิคยูวี-วิสิเบิลและการสังเกตด้วยตาเปล่า พบว่าแคตไอออนที่สามารถเกิดสารประกอบเชิงซ้อนกับเซ็นเซอร์ได้ดีที่สุดคือ คอปเปอร์ไอออน ทั้งนี้ได้ศึกษาความสามารถในการเกิดสารประกอบเชิงซ้อนระหว่างเซ็นเซอร์ **F-mBOH** กับคอปเปอร์ไอออนโดยใช้เทคนิคยูวี-วิสิเบิลไทเทรชัน ได้ค่าคงที่การจับ ($\log \beta$) เท่ากับ 7.36 นอกจากนี้เซ็นเซอร์ **F-mBOH** ยังสามารถสังเกตการเปลี่ยนแปลงสีได้ด้วยตาเปล่าเมื่อเกิดสารประกอบเชิงซ้อนกับคอปเปอร์ไอออนภายในเวลา 10 นาที โดยเปลี่ยนจากสารละลายใสไม่มีสีเป็นสีเหลือง และมีการเปลี่ยนแปลงเป็นสารละลายไม่มีสีเมื่อเติมไซยาไนด์ไอออน ดังนั้นสามารถใช้เซ็นเซอร์ **F-mBOH** ในการตรวจจับคอปเปอร์และไซยาไนด์ไอออนได้ทั้งเทคนิคยูวี-วิสิเบิลและการสังเกตการเปลี่ยนแปลงสีด้วยตาเปล่า

คำสำคัญ: ฟลูออเรสซิน, กรดโบโรนิก, คอปเปอร์, ไซยาไนด์, เซ็นเซอร์

Abstract

A major target of this research is to synthesize and characterize a new sensor containing fluorescein and boronic acid moieties **F-mBOH** for detection of copper and cyanide ions. Complexation studies in acetonitrile of sensor with various cations were evaluated by UV-Vis spectrophotometry and naked-eye detection found that **F-mBOH** was highly selective toward copper ion. The complexation ability with copper ion of **F-mBOH** determined by UV-visible titration exhibited the $\log \beta$ values of 7.36. Interestingly, in the presence of copper ions, the colorless solution of **F-mBOH** changed to strong yellow color within 10 min. Upon the addition of cyanide anions, the strong yellow color faded to colorless immediately. Consequently, **F-mBOH** could be served as excellently naked-eye sensors for detection of copper and cyanide ion.

Keywords: Fluorescein, Boronic acid, Copper, Cyanide, Sensor

บทนำ

ในปัจจุบันนักวิจัยพยายามออกแบบโมเลกุลเซ็นเซอร์และศึกษาถึงความสามารถในการตรวจจับแคตไอออนและแอนไอออนเพราะสามารถนำไปประยุกต์ใช้ตรวจจับแคตไอออนหรือแอนไอออนในเซลล์ของสิ่งมีชีวิตได้ ซึ่งคอปเปอร์ไอออนเป็นไอออนที่นักวิจัยให้ความสำคัญในการศึกษามาก เนื่องจากคอปเปอร์เป็นส่วนประกอบของเอนไซม์บางชนิดในร่างกายมนุษย์ และยังเป็นตัวเร่งปฏิกิริยาที่ช่วยให้เอนไซม์ทำงานได้ดียิ่งขึ้น ในทางตรงข้ามถ้าร่างกายได้รับปริมาณของคอปเปอร์มากเกินไป จะทำให้เกิดโรคทางระบบประสาทได้ เช่น โรคอัลไซเมอร์ โรคพาร์คินสัน เป็นต้น [1] นอกจากนี้แอนไอออนที่มีความสำคัญในระบบ

ชีวภาพคือ ไฮยาไนต์ไอออน เพราะไฮยาไนต์ไอออนถ้าอยู่ในสิ่งมีชีวิตจะส่งผลให้เกิดความเป็นพิษต่อเซลล์ แต่ในทางอุตสาหกรรมได้นำไฮยาไนต์ไปประยุกต์ใช้ให้เกิดประโยชน์ เช่น ใช้ในการสังเคราะห์ไนลอน สังเคราะห์ไฟเบอร์ และเรซิน เป็นต้น [2]

ในหลายงานวิจัยได้ศึกษาเซ็นเซอร์ที่สามารถตรวจวัดคอปเปอร์ โดยใช้การเปลี่ยนแปลงสัญญาณฟลูออเรสเซนซ์ สัญญาณยูวี-วิชิเบิล และสามารถสังเกตการเปลี่ยนแปลงสีด้วยตาเปล่าได้ เนื่องจากวิธีการที่กล่าวมาเป็นวิธีที่ประหยัดต้นทุนในการสร้างเซ็นเซอร์เพื่อใช้ในการตรวจวัดไอออนได้ [3-4] สารประกอบที่สามารถให้สัญญาณฟลูออเรสเซนซ์และยูวี-วิชิเบิลคือ ฟลูออเรสซินหรือโรดามีน ถ้าฟลูออเรส

ซีนหรือโรดามีนอยู่ในรูปปิดวง (Spirolactam หรือ Closed Form) จะไม่สามารถให้สัญญาณฟลูออเรสเซนซ์หรือยูวี แต่ถ้าอยู่ในรูปที่เปิดวง (Opened Form) จะสามารถให้สัญญาณฟลูออเรสเซนซ์และยูวี-วิชิเบิล และทำให้ค่าการดูดกลืนแสงเกิดการเปลี่ยนแปลงซึ่งสามารถสังเกตเห็นการเปลี่ยนแปลงสีได้ด้วยตาเปล่า [5-6] ในปัจจุบันนี้มีผู้ให้ความสนใจศึกษาเซ็นเซอร์ที่มีโรดามีนเป็นส่วนประกอบที่สามารถเปิด-ปิดวงได้ โดยแคตไอออน เช่น Hg^{2+} [7], Cu^{2+} [8], Fe^{3+} [9] แต่สำหรับเซ็นเซอร์ที่มีฟลูออเรสซีนเป็นส่วนประกอบยังมิได้มีผู้สนใจศึกษาน้อยมาก

กรดโบโรนิกเป็นส่วนประกอบที่สำคัญของเซ็นเซอร์หลายชนิด เพราะกรดโบโรนิกสามารถเกิดปฏิกิริยากับสารที่มีไดออลเป็นส่วนประกอบ สามารถนำไปใช้เป็นเซ็นเซอร์ตรวจจบน้ำตาลและคาร์โบไฮเดรตได้ [10] นอกจากนี้กรดโบโรนิกยังมีความสามารถในการเกิดปฏิกิริยากับไฮยาไนด์ไอออน [11] ดังนั้นในงานวิจัยนี้จึงสนใจศึกษาเซ็นเซอร์ที่มีฟลูออเรสซีนเป็นส่วนประกอบที่สามารถเปิด-ปิดวงได้โดยคอปเปอร์ไอออน และได้นำหมู่โบโรนิกมาเชื่อมต่อกับวงฟลูออเรสซีนเพื่อใช้เป็นส่วนที่สามารถตรวจจบบกับไฮยาไนด์ไอออนได้ (**F-mBOH**)

วัตถุประสงค์ของการวิจัย

1. เพื่อสังเคราะห์และพิสูจน์เอกลักษณ์ของเซ็นเซอร์ที่มีอนุพันธ์ของ 4 ฟลูออเรสซีนและกรดโบโรนิกเพื่อใช้เป็นตัวตรวจวัดคอปเปอร์และไฮยาไนด์ไอออน

2. เพื่อศึกษากลไกการเกิดปฏิกิริยาระหว่างเซ็นเซอร์กับคอปเปอร์และไฮยาไนด์ไอออน

อุปกรณ์และวิธีดำเนินการวิจัย

1. การพิสูจน์เอกลักษณ์ของสาร

F-mBOH

ในงานวิจัยนี้ได้ใช้เทคนิค ^1H -NMR, ^{13}C -NMR และ ESI-High Resolution Mass Spectroscopy ในการพิสูจน์เอกลักษณ์ของสาร **F-mBOH** และได้ผลการพิสูจน์เอกลักษณ์ดังนี้

^1H -NMR (400 MHz, $DMSO-d_6$): δ (in ppm)=9.925 (s, -ArOH, 2H), 8.784 (s, -N=CH, 1H), 8.129 (s, -BOH, 2H), 7.918 (J=7.2 Hz, d, -ArH, 1H), 7.757 (J=7.2 Hz, d, -ArH, 2H), 7.600 (m, -ArH, 2H), 7.474 (J=8.4 Hz, d, -ArH, 1H), 7.317 (J=7.2 Hz, t, -ArH, 1H), 7.101 (J=7.6 Hz, d, -ArH, 1H), 6.666 (J=1.6 Hz, d, -ArH, 2H), 6.516 (m, -ArH, 2H), 6.461 (m, -ArH, 2H). ^{13}C NMR (100 MHz, $DMSO-d_6$): δ (in ppm)= 163.349, 158.192, 151.597, 150.475, 148.373, 135.726, 133.617, 133.298, 132.947, 128.648, 128.081, 127.509, 126.966, 123.249, 122.816, 111.991, 109.549, 102.163, 64.623. ESI-HRMS: m/z Calcd for $[M+CH_3OH+H_3O]^+ = 529.1777$, found 529.1552.

2. การศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของเซ็นเซอร์ **F-mBOH** กับแคตไอออนโดยเทคนิคยูวี-วิชิเบิลสเปกโตรโฟโตเมทรี แบบปริมาณแคตไอออนมากเกินพอ

นำเซ็นเซอร์ **F-mBOH** มาศึกษาการเกิดสารประกอบเชิงซ้อนกับแคตไอออนของ Cu^{2+} , Cd^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} , Ag^+ , Mg^{2+} ในตัวทำละลายอะซีโตไนไตรท์ (CH_3CN) ซึ่งมี tetra *n*-butyl ammonium hexafluoro phosphate (Bu_4PF_6) เป็น supporting electrolyte ความเข้มข้น 0.01 M โดยเตรียม stock solution ของ **F-mBOH** ความเข้มข้น 0.001 M และเตรียม stock solution ของแคตไอออนความเข้มข้น 0.01 M ซึ่งในการทดลองจะเตรียมสารละลายใส่ในคิวเวตต์ขนาดเซลล์เท่ากับ 1 cm ความเข้มข้นสุดท้ายของ **F-mBOH** 3×10^{-5} M ปริมาตรรวม 2 mL แล้วเติมแคตไอออนลงไปเป็นจำนวน 70 เท่าของ **F-mBOH**

3. การศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของเซ็นเซอร์ **F-mBOH** กับคอปเปอร์ไอออนโดยเทคนิคยูวี-วิชิเบิลไทเทรชัน และหาค่าคงที่การเกิดสารประกอบเชิงซ้อนของเซ็นเซอร์กับคอปเปอร์ไอออน

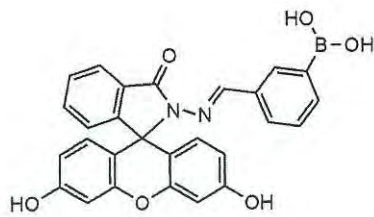
ในการศึกษาจะเตรียม stock solution ของสาร **F-mBOH** เท่ากับ 0.001 M ในขวดวัดปริมาตร 10 mL ในตัวทำละลายอะซีโตไนไตรท์ โดยใช้ supporting electrolyte เหมือนกับการทดลองในหัวข้อ 2 จากนั้นจะเจือจางสารละลายนี้ให้มีความเข้มข้นเท่ากับ 2.5×10^{-5} M ในการไทเทรต ซึ่งได้เตรียมความเข้มข้นของ

คอปเปอร์ไอออน เท่ากับ 0.005 M ในตัวทำละลายอะซีโตไนไตรท์ โดยใช้ Supporting electrolyte เหมือนกับการทดลองในหัวข้อ 2 ในการบันทึกกราฟของยูวี-วิชิเบิล จะบันทึกความยาวคลื่นตั้งแต่ 200-800 nm ที่อุณหภูมิห้อง ซึ่งจะใช้สารละลายของคอปเปอร์เติมลงไปสารละลายเซ็นเซอร์ ที่ความเข้มข้น 2.5×10^{-5} M โดยใช้คิวเวตต์ขนาดเซลล์เท่ากับ 1 cm จากนั้นจะเริ่มบันทึกกราฟของยูวี-วิชิเบิลหลังจากเติมคอปเปอร์ไอออนลงไป 2 นาทีในการหาค่าคงที่ของการเกิดสารประกอบเชิงซ้อน ($\log \beta$) ได้ใช้โปรแกรม Spectfit 32

ผลการวิจัย

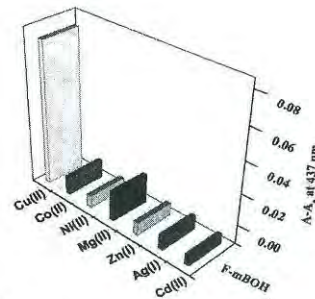
1. การออกแบบเซ็นเซอร์ **F-mBOH**

ในงานวิจัยนี้ได้ออกแบบโมเลกุล **F-mBOH** เพื่อนำมาใช้เป็นเซ็นเซอร์ในการตรวจวัดคอปเปอร์และโซยาไนต์ไอออน โดยขั้นตอนการสังเคราะห์สามารถสังเคราะห์ได้จากการนำฟลูออเรสซินไฮดราไซด์ มาทำปฏิกิริยากับไฮดราซีน [12] จากนั้นนำไปทำปฏิกิริยากับกรดฟอส-มิลฟีนิลโบโรนิก [13] โดยใช้เอทานอลเป็นตัวทำละลาย ได้สารผลิตภัณฑ์ **F-mBOH** เป็นของแข็งสีเหลืองอ่อน ซึ่ง ^{13}C -NMR Spectroscopy ของ **F-mBOH** ปรากฏสัญญาณที่ตำแหน่ง 64.623 ซึ่งเป็นตำแหน่งที่แสดงถึงคาร์บอนของวงสไปโรแลคแทม (spirrolactam) ของ **F-mBOH**

ภาพที่ 1 โมเลกุล **F-mBOH**

2. การศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของเซ็นเซอร์ **F-mBOH** กับแคตไอออนโดยเทคนิคยูวี-วิชิเบิลสเปกโตรโฟโตเมทรี

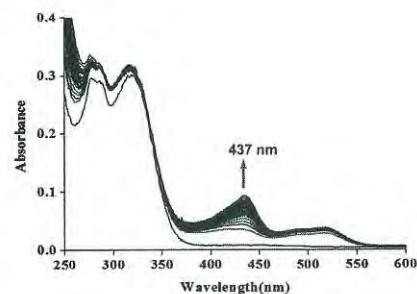
ในขั้นตอนแรกได้นำโมเลกุล **F-mBOH** ไปศึกษาความสามารถในการเกิดสารประกอบเชิงซ้อนกับแคตไอออนชนิดต่างๆ คือ Cu^{2+} , Cd^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} , Ag^+ , Mg^{2+} โดยใช้เทคนิคยูวี-วิชิเบิลสเปกโตรโฟโตเมทรี ผลการทดลองดังแสดงในภาพที่ 2 พบว่า กราฟมีการเปลี่ยนแปลงอย่างชัดเจนเมื่อเติมคอปเปอร์ลงในเซ็นเซอร์ **F-mBOH** โดยกราฟของยูวี-วิชิเบิลที่ตำแหน่งการดูดกลืนแสงความยาวคลื่นที่ 437 nm จะมีการดูดกลืนแสงเพิ่มขึ้น แสดงว่าคอปเปอร์ไอออนช่วยเหนี่ยวนำให้เซ็นเซอร์เปลี่ยนจากรูปปิดวงเป็นเปิดวง ซึ่งเป็นการยืนยันอย่างชัดเจนว่าเซ็นเซอร์ **F-mBOH** มีความจำเพาะเจาะจงกับคอปเปอร์เท่านั้น และได้มีการศึกษาหาค่าคงที่ของการเกิดสารประกอบเชิงซ้อนระหว่าง **F-mBOH** กับคอปเปอร์โดยใช้เทคนิคยูวี-วิชิเบิลไทเทรชัน



ภาพที่ 2 การดูดกลืนแสงของ **F-mBOH** ($3 \times 10^{-5} \text{M}$) กับแคตไอออนชนิดต่างๆ ที่ความยาวคลื่น 437 nm ในอะซิโตนไไตรล์

3. การศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของเซ็นเซอร์ **F-mBOH** กับคอปเปอร์ไอออนโดยเทคนิคยูวี-วิชิเบิลไทเทรชันและหาค่าคงที่ของการเกิดสารประกอบเชิงซ้อนของเซ็นเซอร์กับคอปเปอร์ไอออน

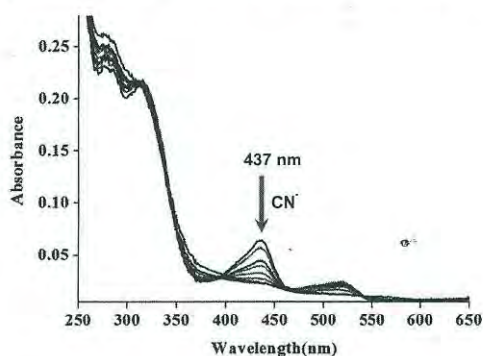
ในการไทเทรตระหว่าง **F-mBOH** กับคอปเปอร์ได้ผลการทดลองดังแสดงในภาพที่ 3 พบว่าสเปกตรัมที่ตำแหน่ง 437 nm มีการดูดกลืนแสงเพิ่มขึ้นแสดงว่า **F-mBOH** อยู่ในรูปเปิดวง



ภาพที่ 3 การไทเทรตระหว่าง **F-mBOH** กับคอปเปอร์ ในอะซิโตนไไตรล์

จากข้อมูลที่บันทึกได้จากกราฟ ภาพที่ 3 สามารถนำไปคำนวณค่าคงที่ในการเกิดสารประกอบเชิงซ้อนได้โดยใช้โปรแกรม Spectfit 32 ซึ่งค่าคงที่ในการเกิดสารประกอบเชิงซ้อน ($\log \beta$) ระหว่าง **F-mBOH** และ คอปเปอร์เท่ากับ 7.36 และคำนวณหาอัตราส่วนในการเกิดสารประกอบเชิงซ้อนระหว่าง **F-mBOH** และคอปเปอร์ได้เท่ากับ 2 ต่อ 1

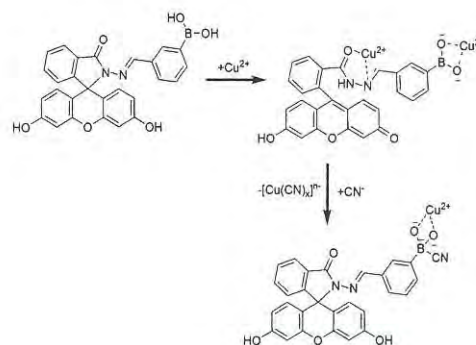
เนื่องจากมีหมู่โบโรนิก ซึ่งสามารถเกิดปฏิกิริยากับไซยาไนด์ไอออนได้ จึงได้มีการทดลองเติมไซยาไนด์ไอออนลงในสารละลายพบว่า สเปกตรัมที่ตำแหน่ง 437 nm มีการดูดกลืนแสงค่อยๆ ลดลง ดังแสดงในภาพที่ 4



ภาพที่ 4 การเติมไซยาไนด์ไอออนเข้าไปในสารละลายที่มีคอปเปอร์อยู่ในสารละลายของ **F-mBOH** (2.5×10^{-5} M)

แสดงว่าไซยาไนด์ไอออนสามารถเกิดปฏิกิริยาได้กับคอปเปอร์ไอออนและคาดว่าไซยาไนด์ไอออนสามารถเข้าไปอยู่ในตำแหน่ง

ที่ว่างของกรดโบโรนิกได้อีกด้วย ซึ่งได้แสดงกลไกในการเกิดปฏิกิริยาระหว่าง **F-mBOH** กับคอปเปอร์และไซยาไนด์ไอออนดังแสดงในภาพที่ 5

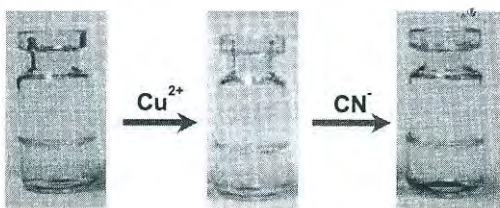


ภาพที่ 5 กลไกในการเกิดปฏิกิริยาระหว่าง **F-mBOH** เมื่อเติมคอปเปอร์และไซยาไนด์ไอออน

เริ่มต้นคอปเปอร์ไอออนสามารถจับกับ **F-mBOH** ได้สองตำแหน่งคือ คอปเปอร์สามารถช่วยเหนี่ยวนำให้วงสไปโรแลคแทม (spirolactam) ของ **F-mBOH** เปิดวงออกได้ ทำให้สามารถสังเกตเห็นการเปลี่ยนแปลงสีได้ โดยจะเปลี่ยนจากไม่มีสีเป็นสีเหลือง และอีกตำแหน่งหนึ่ง คือ คอปเปอร์ไอออนสามารถเกิดสารประกอบเชิงซ้อนที่ตำแหน่งกรดโบโรนิก และเมื่อเติมไซยาไนด์ไอออนเข้าไปในสารละลายไซยาไนด์จะดึงคอปเปอร์ไอออนออกมาแล้วเกิดเป็นสารประกอบเชิงซ้อนของคอปเปอร์ไซยาไนด์

จากกลไกที่เกิดขึ้นคาดว่าไซยาไนด์ไอออนสามารถเกิดปฏิกิริยากับคอปเปอร์ไอออนได้ที่ตำแหน่งสไปโรแลคแทม และทำให้วงสไปโรแลคแทมของ **F-mBOH** เปลี่ยนจากเปิดวงกลับไปเป็นแบบปิดวง ทำให้สีของสารละลายเปลี่ยนจากสีเหลืองกลายเป็นใสไม่มีสี และสาเหตุที่ไซยาไนด์ไม่ดึงคอปเปอร์ที่ตำแหน่งกรดโบโรนิกออกไปได้นั้น อาจเนื่องมาจากความแข็งแรงของพันธะที่ตำแหน่งนี้แข็งแรงกว่าบริเวณวงสไปโรแลคแทม ทั้งนี้กรดโบโรนิกยังมีที่ว่างอีกหนึ่งตำแหน่งที่ไซยาไนด์สามารถเข้าไปเกิดปฏิกิริยาได้ ดังแสดงไว้ในภาพที่ 5

นอกจากนี้ยังสามารถสังเกตการเปลี่ยนแปลงสีได้ด้วยตาเปล่า เมื่อเติมคอปเปอร์ไอออนลงไป จะมีการเปลี่ยนแปลงสีของสารละลายจากไม่มีสีเป็นสีเหลือง และเมื่อเติมไซยาไนด์ลงไปจะทำให้สีของสารละลายเปลี่ยนเป็นไม่มีสีเหมือนเดิม ดังที่ได้แสดงในภาพที่ 6



ภาพที่ 6 การเปลี่ยนแปลงสีของ **F-mBOH** (2.5×10^{-5} M) เมื่อเติมคอปเปอร์และไซยาไนด์ไอออน

สรุปและอภิปรายผลการวิจัย

ผู้วิจัยได้นำเซ็นเซอร์ **F-mBOH** ที่สังเคราะห์ได้ มาศึกษาความสามารถในการเกิดสารประกอบเชิงซ้อนกับแคตไอออนชนิดต่างๆ คือ Cu^{2+} , Cd^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} , Ag^+ และ Mg^{2+} โดยได้ศึกษาในแบบแคตไอออนปริมาณมากเกินพอ ในอะซิโตนไนไตร (CH_3CN) โดยใช้เทคนิคยูวี-วิสิเบิล สเปกโตรโฟโตเมตรี พบว่าเซ็นเซอร์มีความจำเพาะเจาะจงกับคอปเปอร์ไอออนมากที่สุด และจากเทคนิคยูวี-วิสิเบิล ไทเทรชัน จึงสามารถหาความสามารถในการเกิดสารประกอบเชิงซ้อนระหว่างเซ็นเซอร์ **F-mBOH** กับคอปเปอร์ไอออนได้ค่าคงที่ในการเกิดสารประกอบเชิงซ้อน ($\log \beta$) เท่ากับ 7.36 นอกจากนี้เซ็นเซอร์ **F-mBOH** ยังสามารถสังเกตการเปลี่ยนแปลงสีได้ด้วยตาเปล่าเมื่อเกิดสารประกอบเชิงซ้อนกับคอปเปอร์ไอออนภายในเวลา 10 นาที โดยเปลี่ยนจากสารละลายใสไม่มีสีเป็นสีเหลือง และมีการเปลี่ยนแปลงเป็นสารละลายไม่มีสีเมื่อเติมไซยาไนด์ไอออน ดังนั้นสามารถใช้เซ็นเซอร์ **F-mBOH** เป็นตัวตรวจจับคอปเปอร์และไซยาไนด์ไอออนได้ทั้งเทคนิคยูวี-วิสิเบิลและการเปลี่ยนแปลงสีด้วยตาเปล่า

กิตติกรรมประกาศ

ขอขอบคุณโครงการโครงการพัฒนา
อัจฉริยภาพทางวิทยาศาสตร์และเทคโนโลยี
สำหรับเด็กและเยาวชน (JSTP) ที่ให้ทุน
สนับสนุนในการดำเนินการวิจัยในครั้งนี้

ขอขอบพระคุณหน่วยวิจัยเคมีซูปโมเลกุล
(Supramolecular Chemistry Research Unit)
ภาควิชาเคมี จุฬาลงกรณ์มหาวิทยาลัย ที่ได้
เอื้อเฟื้อเครื่องมือ อุปกรณ์และสารเคมีที่จำเป็น
ต่างๆที่ทำให้การวิจัยสำเร็จและเป็นไปได้ด้วยดี

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การแยกประเภทของนิวคลีโอไทด์โดยใช้เซนเซอร์แอเรย์ ผ่านวิธีวิเคราะห์องค์ประกอบหลัก

DISCRIMINATION OF NUCLEOTIDES IN AQUEOUS SOLUTION BY THE SENSOR ARRAYS VIA PCA METHOD

จิราภรณ์ ไชยแจ่ม, คณิศ วังษ์วี, บุษยรัตน์ ธรรมพัฒน์กิจ*
*Chiraporn Chaicham, Kanet Wongrawee, Boosayarat Tomapatanaget**

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บทคัดย่อ

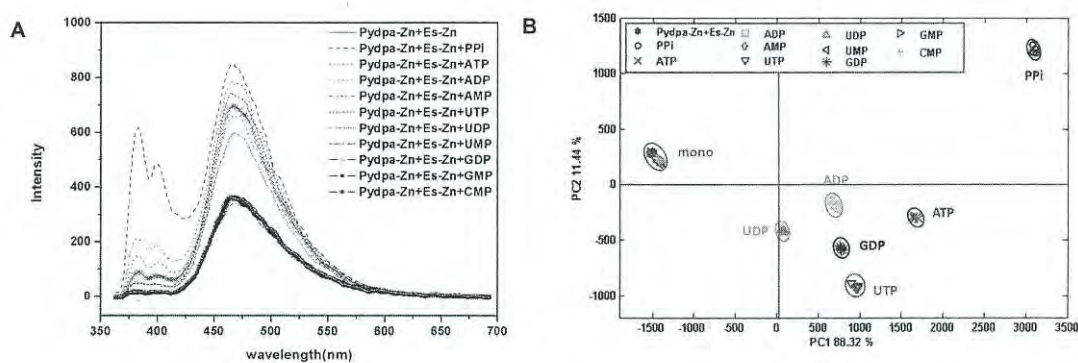
ในงานวิจัยนี้มีจุดมุ่งหมายเพื่อการออกแบบและสังเคราะห์โมเลกุลเซนเซอร์ที่มีอนุพันธ์ของไพรีนและคูมารินเป็นองค์ประกอบ (Pydpa และ Es) สำหรับใช้ในการแยกประเภทของนิวคลีโอไทด์โดยใช้การวิเคราะห์องค์ประกอบหลัก (Principal Component Analysis, PCA) ในขั้นต้นได้เตรียมสารประกอบเชิงซ้อนของเซนเซอร์โมเลกุล Pydpa-Zn, Pydpa-Cu, Es-Zn และ Es-Cu เพื่อที่จะใช้จับกับนิวคลีโอไทด์ ซึ่งหมู่ฟอสเฟตของนิวคลีโอไทด์ สามารถเกิดอันตรกิริยาทางไฟฟ้าสถิตกับสารประกอบเชิงซ้อนของเซนเซอร์โมเลกุลได้ จากนั้นได้ศึกษาความสามารถในการเกิดสารประกอบเชิงซ้อน โดยใช้เทคนิคฟลูออเรสเซนซ์สเปกโทรโฟโตเมทรี พบว่า Pydpa-Zn, Es-Zn และ Es-Cu มีความจำเพาะต่อ PPI ได้ค่าคงที่การจับ ($\log K$) ของ Pydpa-Zn, Es-Zn และ Es-Cu กับ PPI เท่ากับ 8.83, 5.23 และ 4.64 ตามลำดับ นอกจากนี้ได้นำ PCA มาใช้แยกประเภทของนิวคลีโอไทด์ ซึ่งเป็นที่น่าสนใจว่าข้อมูลที่ได้จากการใช้เซนเซอร์แอเรย์ Pydpa-Zn / Es-Zn หรือ Es-Zn / Es-Cu พบว่า สามารถแยกแยะกลุ่มของนิวคลีโอไทด์ ATP, UTP, ADP, UDP และ GDP ได้อย่างชัดเจน

คำสำคัญ: การวิเคราะห์องค์ประกอบหลัก นิวคลีโอไทด์ เซนเซอร์

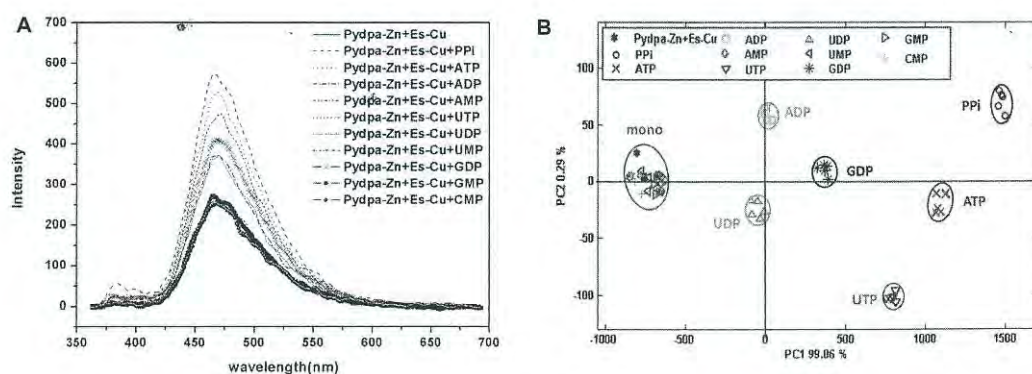
ซึ่งเป็นตำแหน่งของ **Pydpa-Zn** และ **Es-Zn** ตามลำดับ เมื่อเติมนิวคลีโอไทด์ลงไปพบว่า **PPi** จะทำให้เกิดการเปลี่ยนแปลงสัญญาณฟลูออเรสเซนซ์ของทั้งสองฟิคอย่างชัดเจน ในขณะที่นิวคลีโอไทด์อื่นๆ จะให้สัญญาณฟลูออเรสเซนซ์เล็กน้อยแสดงดังรูป 7A สำหรับเซนเซอร์ **Pydpa-Zn+Es-Cu** จะเกิดการเปลี่ยนแปลงสัญญาณฟลูออเรสเซนซ์เฉพาะฟิคที่ 468 nm เมื่อเติมนิวคลีโอไทด์ลงไปในสารละลาย ซึ่งเป็นตำแหน่งของ **Es-Cu** แสดงดังภาพที่ 8A

นอกจากนี้ผลจาก PCA plot ของ **Pydpa-Zn+Es-Zn** สามารถแยกประเภทของนิวคลีโอไทด์ได้

เป็น 3 กลุ่ม ได้แก่ **PPi**, ไต-ไตรฟอสเฟต (**ATP**, **ADP**, **UTP**, **UDP** และ **GDP**) และมอนอฟอสเฟต (**AMP**, **UMP**, **GMP** และ **CMP**) แสดงดังภาพที่ 7B สำหรับกรณีของ **Pydpa-Zn+Es-Cu** ผลจาก PCA plot สามารถแยกแยะประเภทของนิวคลีโอไทด์ได้อย่างชัดเจน ได้แก่ **PPi**, **ATP**, **ADP**, **UTP**, **UDP** และ **GDP** แสดงดังภาพที่ 8B จากผลการทดลองทั้งหมดพบว่า เซนเซอร์ทั้งหมดชอบที่จะจับกับฟอสเฟตตั้งแต่ 2 หน่วยขึ้นไป จึงทำให้เซนเซอร์ทุกตัวไม่สามารถจับกับกลุ่มของมอนอฟอสเฟตได้



ภาพที่ 7 A) ฟลูออเรสเซนซ์สเปกตรัมและ B) PCA plot ของ **Pydpa-Zn+Es-Zn**



ภาพที่ 8 A) ฟลูออเรสเซนซ์สเปกตรัมและ B) PCA score plot ของ **Pydpa-Zn+Es-Cu**

สรุปและอภิปรายผล

ผู้วิจัยประสบผลสำเร็จในการสังเคราะห์ **Pydpa-Zn** และ **Pydpa-Cu** ในการศึกษาการเกิดสารประกอบเชิงซ้อนระหว่าง **Esculetin** กับโลหะแทรนซิชัน Zn^{2+} และ Cu^{2+} โดยใช้เทคนิคยูวี-วิสิเบิลสเปกโทรสโกปีพบว่าอัตราส่วนในการเกิดสารประกอบเชิงซ้อนเป็น 2:1 ระหว่าง **Esculetin** กับ Zn^{2+} และ Cu^{2+} จากนั้นนำเซนเซอร์ **Pydpa-Zn**, **Pydpa-Cu**, **Es-Zn** และ **Es-Cu** ที่สังเคราะห์ได้มาศึกษาการเกิดสารประกอบเชิงซ้อนกับนิวคลีโอไทด์ชนิดต่างๆ ซึ่งจากผลการทดลองพบว่าเซนเซอร์ **Pydpa-Zn**, **Es-Zn** และ **Es-Cu** มีความจำเพาะต่อ PPI แต่สำหรับนิวคลีโอไทด์อื่นๆ จะใช้การวิเคราะห์องค์ประกอบหลัก (Principal component analysis, PCA) ช่วยในการแยกประเภทของนิวคลีโอไทด์ โดยการสร้างเป็นเซนเซอร์แอรเรย์ ซึ่งจะใช้ข้อมูลในการเกิดสารประกอบเชิงซ้อนของเซนเซอร์แต่ละชนิดกับนิวคลีโอไทด์ พบว่า เพียงสองเซนเซอร์ก็สามารถแยกนิวคลีโอไทด์อื่นๆ ออกจากกันได้อย่างชัดเจน คือ **Pydpa-Zn / Es-Zn** หรือ **Es-Zn / Es-Cu**

ส่วนกรณีของการสร้างเซนเซอร์ใหม่ระหว่าง **Pydpa-Zn** และ **Es-Cu** (**Pydpa-Zn+Es-Cu**) สามารถแยกแยะประเภทของนิวคลีโอไทด์ได้อย่างชัดเจน คือ PPI, ATP, ADP, UTP, UDP และ GDP นอกจากนี้ในการศึกษาค่าคงที่ในการเกิดสารประกอบเชิงซ้อนระหว่างเซนเซอร์กับ PPI ($\log K$) เท่ากับ 8.83, 5.23 และ 4.64 สำหรับ **Pydpa-Zn**, **Es-Zn** และ **Es-Cu** กับ PPI ตามลำดับ

กิตติกรรมประกาศ

ขอขอบคุณโครงการพัฒนากำลังคนด้านวิทยาศาสตร์

(ทุนเรียนดีวิทยาศาสตร์แห่งประเทศไทย) ที่ให้ทุนการศึกษา และขอขอบคุณ สกว. และ สกอ. (RMU 5380003) ในการให้ทุนสนับสนุนการวิจัยในครั้งนี้ ขอขอบพระคุณหน่วยวิจัยเคมีซูปรา (Supramolecular Chemistry Research Unit) ภาควิชาเคมี จุฬาลงกรณ์มหาวิทยาลัย ที่ได้เอื้อเฟื้ออุปกรณ์ เครื่องมือ และสารเคมีที่จำเป็นต่างๆ ที่ทำให้การวิจัยสำเร็จไปได้ด้วยดี

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Abstract

This research aims to design and synthesize sensors containing pyrene and coumarin derivatives (**Pydpa** and **Es**) and then apply the sensory molecule to discriminate various nucleotides in aqueous solution by Principle Component Analysis (PCA). Initially, the complexation of **Pydpa-Zn**, **Pydpa-Cu**, **Es-Zn**, and **Es-Cu** were prepared as the sensory molecules to coordinate the phosphate anion based nucleotides undergone the electrostatic interaction. The complexation studies of sensors were carried out by Fluorescence Spectrophotometry. It was found that **Pydpa-Zn**, **Es-Zn**, and **Es-Cu** showed selectively to PPI. The log *K* values of **Pydpa-Zn**, **Es-Zn**, and **Es-Cu** towards PPI were 8.83, 5.23 and 4.64, respectively. In addition, PCA analysis was applied to classify the types of nucleotides. Interestingly, the combinatorial data obtained by the sensor arrays of **Pydpa-Zn** / **Es-Zn** or **Es-Zn** / **Es-Cu** in the attribution of PCA method could obviously discriminate ATP, UTP, ADP, UDP and GDP.

Keywords: Principal Component Analysis (PCA), Nucleotide, Sensor

บทนำ

นิวคลีโอไทด์เป็นโครงสร้างพื้นฐานของดีเอ็นเอที่พบในสิ่งมีชีวิต และมีความสำคัญต่อการเจริญเติบโต การทำงานของอวัยวะต่างๆ อีกทั้งมีหน้าที่ซ่อมแซมเซลล์ที่ถูกทำลาย รวมถึงการเผาผลาญพลังงานในระดับเซลล์ ดังนั้นการตรวจวัดโมเลกุลนิวคลีโอไทด์จึงเป็นกุญแจสำคัญสำหรับตรวจวินิจฉัยโรค [1] ในปัจจุบันการออกแบบและสังเคราะห์โมเลกุลเซนเซอร์สำหรับตรวจวัดสารจำพวกนิวคลีโอไทด์นั้น จะพิจารณาจากโครงสร้างพื้นฐานของ นิวคลีโอไทด์ซึ่งประกอบด้วย 3 ส่วนหลักๆ คือ น้ำตาล เบส และหมู่ฟอสเฟต จากงานวิจัยที่ผ่านมาพบว่า หมู่ฟอสเฟตสามารถจับกับโลหะสังกะสี [2] และคอปเปอร์ [3] ได้ดี จึงนำเอาสารประกอบเชิงซ้อนของโลหะคอปเปอร์หรือสังกะสีมาใช้ตรวจวิเคราะห์นิวคลีโอไทด์ แล้วติดตามการเปลี่ยนแปลงสีของสารละลาย [4] หรือสัญญาณฟลูออเรสเซนซ์ [5-6] อย่างไรก็ตามการตรวจวัดนิวคลีโอไทด์ด้วยสารประกอบเชิงซ้อนของคอปเปอร์หรือสังกะสีนั้น จะให้ความจำเพาะกับหมู่ฟอสเฟตเพียงหมู่เดียวส่งผลให้ไม่สามารถจำแนกชนิดของ

นิวคลีโอไทด์ได้ [4] เพื่อให้มีความจำเพาะเจาะจงต่อชนิดของนิวคลีโอไทด์เพิ่มขึ้น จึงได้มีการพัฒนาการตรวจวัดโดยใช้เซนเซอร์มากกว่าหนึ่งชนิดมารวมวิเคราะห์กลุ่มของสารตัวอย่าง [7] ซึ่งเทคนิคนี้เรียกว่า “เซนเซอร์แอเรย์” เพื่อความสะดวกในการวิเคราะห์ด้วยเทคนิคนี้ ได้มีการนำเอากระบวนการทางคณิตศาสตร์มาช่วยวิเคราะห์ ซึ่งกระบวนการทางคณิตศาสตร์นี้คือ การวิเคราะห์องค์ประกอบหลัก (Principal Component Analysis, PCA)

ในปัจจุบันมีผู้ให้ความสนใจในกระบวนการนี้เป็นอย่างมาก เนื่องจากสามารถใช้แยกประเภทของสารที่มีลักษณะคล้ายคลึงกัน เช่น โลหะ [7-9] และโปรตีน [10] หรือแม้กระทั่งนิวคลีโอไทด์ ยกตัวอย่างเช่น ในงานวิจัยของ Severin [11] ได้ศึกษาการตรวจวัดนิวคลีโอไทด์โดยอาศัยการแทนที่อินดิเคเตอร์ (multicomponent indicators displacement assay, MIDA) อินดิเคเตอร์ที่ใช้จะต้องมีความสามารถในการจับกับสารประกอบเชิงซ้อนโรเดียมได้แตกต่างกัน ซึ่งให้การดูดกลืนแสงที่แตกต่างกันด้วย แต่อย่างไรก็ตามการ

วิเคราะห์ด้วยเทคนิคยูวี-วิสิเบิลนั้น มีข้อจำกัดคือ สีของสารตัวอย่างจะมีผลต่อการวิเคราะห์ทำให้เกิดความคลาดเคลื่อนได้ ดังนั้นในงานวิจัยนี้ ผู้วิจัยจึงสนใจศึกษาการตรวจวัดนิวคลีโอไทด์ โดยใช้กระบวนการทางคณิตศาสตร์ เพื่อแยกประเภทของนิวคลีโอไทด์ โดยสร้างเป็นเซนเซอร์แอเรีย ซึ่งจะใช้ข้อมูลที่ได้จากผลการทดลองทางฟลูออเรสเซนส์สเปกโทรสโกปี โดยใช้เซนเซอร์ **Pydpa-Zn**, **Pydpa-Cu**, **Es-Zn** และ **Es-Cu**

วัตถุประสงค์ของการวิจัย

เพื่อใช้ในการแยกแยะประเภทของนิวคลีโอไทด์ โดยสร้างเป็นเซนเซอร์แอเรีย ซึ่งจะอาศัยกระบวนการคำนวณทางคณิตศาสตร์ในการวิเคราะห์

วิธีดำเนินการวิจัย

1. การสังเคราะห์เซนเซอร์ **Pydpa-Zn** และ **Pydpa-Cu**

1.1 การเตรียมสาร **Pydpa**

Pydpa สังเคราะห์ในทำนองเดียวกับงานวิจัยของ Lucy A. Mullice [12] โดยเริ่มจากละลาย pyrenecarboxaldehyde 0.23 g, dipicolylamine 0.20 g และ sodium tris(acetoxy) borohydride 0.28 g ในตัวทำละลายไดคลอโรมีเทน 10 mL คนสารละลายผสมภายใต้บรรยากาศไนโตรเจนที่อุณหภูมิห้องเป็นเวลา 5 ชั่วโมง เติมน้ำ sodium hydrogen carbonate เพื่อปรับให้สารละลายเป็นกลาง และสกัดด้วยไดคลอโรมีเทน 3 ครั้ง ครั้งละ 30 mL กำจัดน้ำด้วย anhydrous sodium sulfate และระเหยตัวทำละลายให้แห้ง ทำผลิตภัณฑ์ให้บริสุทธิ์ด้วยเทคนิคคอลัมน์โครมาโทกราฟี โดยใช้ 5% เมทานอลในไดคลอโรมีเทน จะได้ของเหลวสีเหลืองของ **Pydpa** (0.36 g, 88%)

$^1\text{H-NMR}$ (400 MHz, DMSO- d_6) : δ (in ppm) = 8.50 (d, J = 4.0 Hz, 2H-pyridine), 8.37 (d, J = 8.8 Hz, 1H-phenanthrene),

8.27 (s, 1H-phenanthrene), 8.25-8.01 (m, 7H-phenanthrene), 7.70 (t, J = 7.8 Hz, 2H-pyridine), 7.48 (d, J = 8.0 Hz, 2H-pyridine), 7.21 (t, J = 10.0 Hz, 2H-pyridine), 4.35 (s, 2H-methylrine), 3.81 (s, 4H-methylrine). $^{13}\text{C-NMR}$ (400 MHz, DMSO- d_6) : δ (in ppm) = 120.9, 148.6, 136.2, 124.1, 156.1, 60.1, 58.1, 127.0, 126.0, 126.6, 125.7, 126.3, 128.3, 133.4, 124.8, 124.5, 124.2, 134.9, 132.8 ESI-HRMS: calcd for $\text{C}_{29}\text{H}_{23}\text{N}_3$ m/z $[\text{M}+\text{H}]^+$ 414.2048, found 414.2022. UV-Visible (DMSO): 315, 330, 347 nm. Fluorescence (DMSO): 379, 398 nm. ESI-HRMS: calcd for $\text{C}_{29}\text{H}_{23}\text{N}_3$ m/z $[\text{M}+\text{H}]^+$ 414.2048, found 414.2022.

1.2 การเตรียมสาร **Pydpa-Zn**

ละลาย Zn (ClO_4) $_2 \cdot 6\text{H}_2\text{O}$ 0.171 g ในตัวทำละลายเมทานอล 5 mL เติมลงในสารละลาย **Pydpa** 0.112 g ในตัวทำละลายเมทานอล 5 mL คนสารละลายผสมภายใต้บรรยากาศไนโตรเจนที่อุณหภูมิห้องเป็นเวลา 24 ชั่วโมง ต่อก่อนสีเหลืองอ่อนตกออกมา จากนั้นกรองตะกอนและล้างตะกอนด้วยเมทานอลเย็น จะได้ **Pydpa-Zn** (0.21 g, 100%)

$^1\text{H-NMR}$ spectrum (400 MHz, DMSO- d_6) : δ (in ppm) = 8.71 (s, 2H-pyridine), 8.45-8.13 (m, 9H-phenanthrene), 7.94 (d, J = 9.2 Hz, 2H-pyridine), 7.68 (t, J = 6 Hz, 2H-pyridine), 7.56 (d, J = 6.4 Hz, 2H-pyridine), 4.55 (s, 2H-methylene), 4.35 (d, J = 15.2 Hz, 2H-methylene), 3.69 (d, J = 15.6 Hz, 2H-methylene). $^{13}\text{C-NMR}$ spectrum (400 MHz, DMSO- d_6) : δ (in ppm) = 154.1, 147.6, 140.5, 130.9, 130.7, 130.6, 130.5, 129.9, 128.0, 127.9, 127.2, 126.4, 125.8, 125.7, 125.3, 124.8, 124.7, 124.6, 124.0, 123.7, 122.6, 55.6, 51.9 UV-Visible (DMSO): 315, 330, 347

nm. Fluorescence (DMSO): 379, 396 nm. ESI HRMS: calcd for $C_{29}H_{23}N_3Cl_3O_{12}Zn$ m/z $[M+(ClO_4)_3]^-$ 773.9639, found 773.9829.

1.3 การเตรียมสาร Pydpa-Cu

ละลาย $CuCl_2 \cdot 2H_2O$ 0.05 g ในตัวทำละลายเมทานอล 5 mL เติมลงในสารละลาย Pydpa 0.078 g ในตัวทำละลายเมทานอล 5 mL คนสารละลายผสมภายใต้บรรยากาศไนโตรเจนที่อุณหภูมิห้องเป็นเวลา 24 ชั่วโมง ตะกอนสีเขียวอ่อนจะตกออกมา จากนั้นกรองตะกอนและล้างตะกอนด้วยเมทานอลเย็น จะได้ Pydpa-Cu (0.90 g, 100%)

UV-Visible (DMSO): 320, 333, 350 nm. Fluorescence (DMSO): 384, 402, 475 nm. ESI-HRMS: calcd for $C_{29}H_{23}N_3$ m/z $[M+H]^+$ 569.1320, found 568.9213.

2. การศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของเซนเซอร์ Esculetin กับแคตไอออน (Zn^{2+} และ Cu^{2+}) โดยเทคนิคยูวี-วิสิเบิล ไทเทรชัน

ในการศึกษาจะเตรียมสารละลายตั้งต้นของสาร Esculetin ที่ความเข้มข้น 0.001 M ในขวดวัดปริมาตร 5 mL ใน DMSO และเจือจางสารละลายนี้ด้วยบัฟเฟอร์ HEPES ความเข้มข้น 0.01 M pH 7.4 สำหรับใช้ในการไทเทรต ให้มีความเข้มข้น 5×10^{-5} M เตรียมความเข้มข้นของ Zn^{2+} และ Cu^{2+} เท่ากับ 0.001 M ในการบันทึกกราฟของยูวี-วิสิเบิล บันทึกความยาวคลื่นตั้งแต่ 200-800 nm ที่อุณหภูมิห้อง โดยใช้คิวเวตต์ขนาดเซลล์เท่ากับ 1 cm จากนั้นจะเริ่มบันทึกกราฟของยูวีวิสิเบิลหลังจากเติมแคตไอออนลงไป 1 นาที

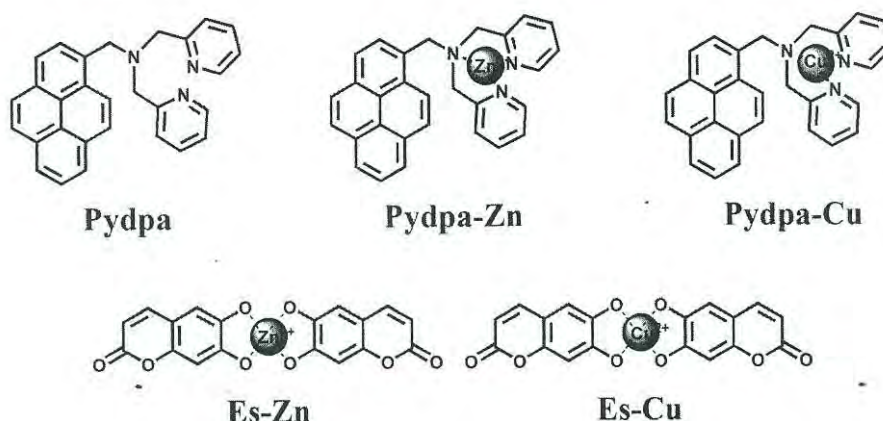
3. การศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของเซนเซอร์ Pydpa-Zn, Pydpa-Cu, Es-Zn และ Es-Cu กับ นิวคลีโอไทด์ ด้วยเทคนิคฟลูออเรสเซนซ์ สเปกโตรโฟโตเมตรี

เตรียมสารละลายตั้งต้นของสาร Pydpa-Zn และ Pydpa-Cu เท่ากับ 0.001 M และ stock solution ของสาร Es-Zn และ Es-Cu ความเข้มข้น 5×10^{-4} M ในขวดวัดปริมาตร 5 mL เตรียม stock solution ของนิวคลีโอไทด์ (PPI, ATP, ADP, AMP, UTP, UDP, UMP, GDP, GMP, CMP) ความเข้มข้น 0.001 M ในสารละลายบัฟเฟอร์ HEPES ความเข้มข้น 0.01 M pH 7.4 ในการทดลองจะเตรียมสารละลายใส่ในคิวเวตต์ขนาดเซลล์เท่ากับ 1 cm ความเข้มข้นสุดท้ายของ Pydpa-Zn, Pydpa-Cu, Es-Zn และ Es-Cu เท่ากับ 1×10^{-5} M ปริมาตรรวม 2 mL เติมนิวคลีโอไทด์ลงไปเป็น 10 เท่าของ Pydpa-Zn, Pydpa-Cu, Es-Zn และ Es-Cu นำข้อมูลที่ได้มาหาค่าคงที่ของการเกิดสารประกอบเชิงซ้อน ($\log K$) ระหว่างเซนเซอร์กับ PPI โดยใช้โปรแกรม Solver

ผลการวิจัย

การออกแบบเซนเซอร์ Pydpa-Zn, Pydpa-Cu, Es-Zn และ Es-Cu

ในงานวิจัยนี้ได้ออกแบบเซนเซอร์ Pydpa-Zn, Pydpa-Cu, Es-Zn และ Es-Cu เพื่อใช้ในการตรวจวัดนิวคลีโอไทด์ แสดงดังภาพที่ 1 การสังเคราะห์ Pydpa สามารถสังเคราะห์ได้จากการนำโมเลกุลไพรีนอัลดีไฮด์ทำปฏิกิริยากับไดฟีโคลิลเอมีน จากนั้นนำ Pydpa ที่สังเคราะห์ได้มาเตรียมสารประกอบเชิงซ้อนกับซิงค์และคอปเปอร์ไอออน ได้ผลิตภัณฑ์ของ Pydpa-Zn และ Pydpa-Cu เป็นของแข็งสีเหลืองอ่อนและสีเขียวอ่อนตามลำดับ

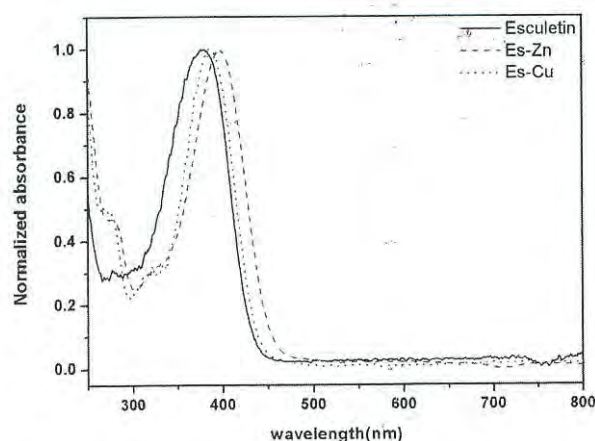


ภาพที่ 1 โมเลกุล Pydpa, Pydpa-Zn, Pydpa-Cu, Es-Zn และ Es-Cu

การศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของเซนเซอร์ **Esculetin** กับแคตไอออน (Zn^{2+} และ Cu^{2+}) โดยเทคนิคยูวี-วิสิเบิล สเปกโตรโฟโตเมทรี

Esculetin (Es) มีหมู่ไคโอรเป็นองค์ประกอบซึ่งสามารถเกิดสารประกอบเชิงซ้อนกับไอออนของโลหะแทรนซิชันได้ ดังนั้นจึงได้ศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของ **Esculetin** กับซิงค์และคอปเปอร์ไอออน โดยใช้เทคนิคยูวี-วิสิเบิล สเปกโตรโฟโตเมทรี โดย **Esculetin** จะให้ค่าการดูด

กลืนแสงที่ความยาวคลื่น 380 nm จากผลการทดลองจะพบการเปลี่ยนแปลงการดูดกลืนคลื่นแสงเคลื่อนไปทางความยาวคลื่นสูงขึ้น (red shift) อย่างชัดเจนเมื่อเติมซิงค์และคอปเปอร์ไอออนลงไปในสารละลาย **Esculetin** ซึ่งจะพบการดูดกลืนแสงของ **Es-Zn** และ **Es-Cu** ที่ความยาวคลื่น 398 nm และ 387 nm ตามลำดับ แสดงให้เห็นว่า **Esculetin** สามารถเกิดสารประกอบเชิงซ้อนกับซิงค์และคอปเปอร์ไอออนได้

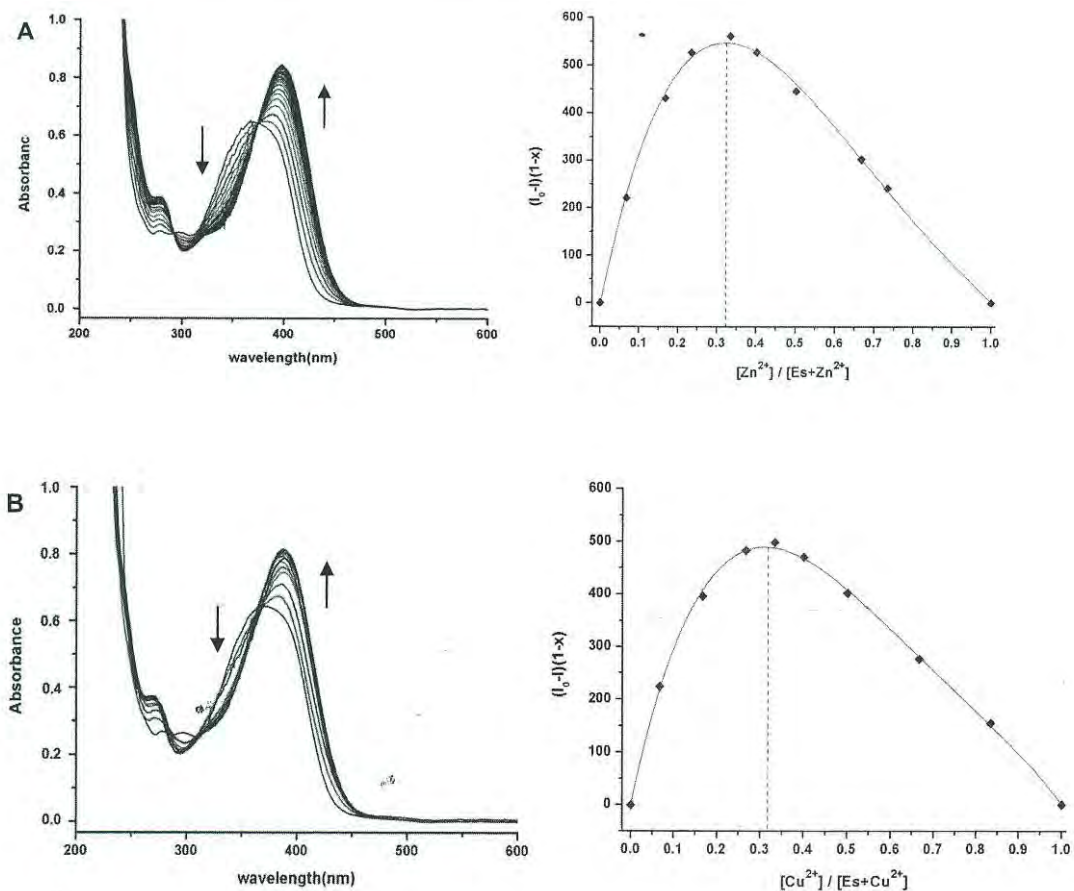


ภาพที่ 2 การดูดกลืนแสงของ **Es-Zn** และ **Es-Cu** (1×10^{-5} M) ที่ความยาวคลื่น 398 nm และ 387 nm ตามลำดับ ในสารละลายบัฟเฟอร์ HEPES 0.01 M pH 7.4

การศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของเซนเซอร์ **Esculetin** กับแคตไอออน (Zn^{2+} และ Cu^{2+}) โดยเทคนิค ยูวี-วิสิเบิล โทเทรชัน และศึกษาอัตราส่วนในการเกิดสารประกอบเชิงซ้อนระหว่าง **Esculetin** กับแคตไอออน

การไทเทรตระหว่าง **Esculetin** กับแคตไอออน (Zn^{2+} และ Cu^{2+}) ได้ผลการทดลองแสดงในภาพที่ 3

พบการเปลี่ยนแปลงค่าการดูดกลืนแสงของ **Es-Zn** และ **Es-Cu** ที่ความยาวคลื่น 398 nm และ 387 nm ตามลำดับ นอกจากนี้อัตราส่วนในการเกิดสารประกอบเชิงซ้อนระหว่าง **Esculetin** กับแคตไอออน (Zn^{2+} และ Cu^{2+}) เท่ากับ 2 ต่อ 1 ซึ่งสอดคล้องกับงานวิจัยที่ผ่านมา [2]



ภาพที่ 3 การไทเทรตและอัตราส่วนในการเกิดสารประกอบเชิงซ้อนระหว่าง **Esculetin** กับซิงค์ (A) และ คอปเปอร์ไอออน (B) ในบัฟเฟอร์ HEPES 0.01 M pH 7.4

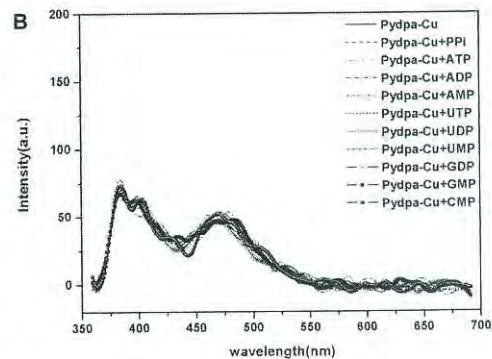
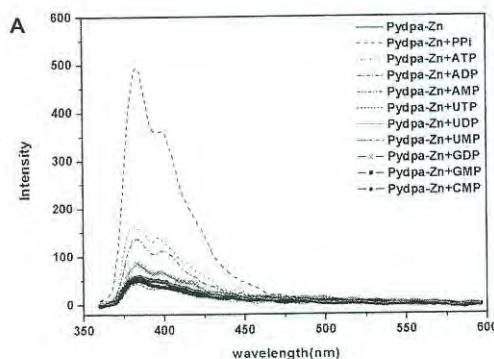
การศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของเซนเซอร์ Pydpa-Zn, Pydpa-Cu, Es-Zn และ Es-Cu กับนิวคลีโอไทด์ โดยเทคนิคฟลูออเรสเซนซ์ สเปกโตรโฟโตเมทรี

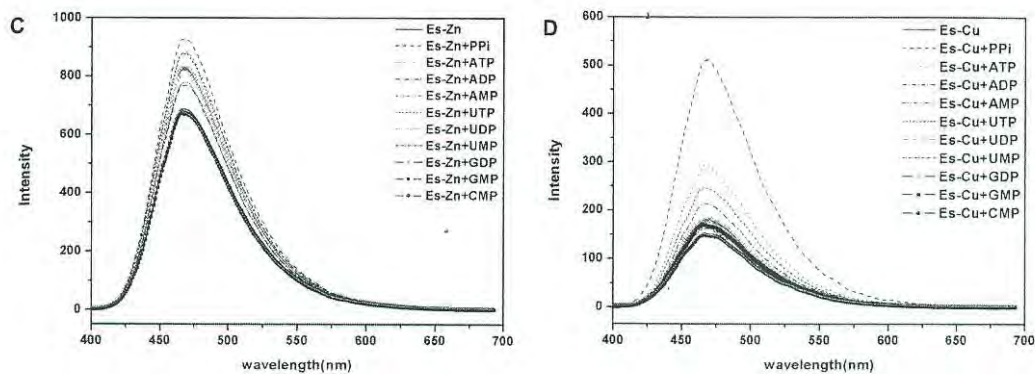
จากงานวิจัยที่ผ่านมาพบว่า ไอออนของโลหะแทรนซิชันโดยเฉพาะซิงค์และคอปเปอร์สามารถโคออร์ดิเนตกับหมู่ฟอสเฟสของนิวคลีโอไทด์ได้ [13] ดังนั้น การศึกษาสมบัติการเกิดสารประกอบเชิงซ้อนของเซนเซอร์ Pydpa-Zn, Pydpa-Cu, Es-Zn และ Es-Cu กับนิวคลีโอไทด์ แสดงดังภาพที่ 4

เซนเซอร์ Pydpa-Zn จะเกิดการคายแสงที่ความยาวคลื่น 384 nm และเมื่อเติม PPI ลงไปในสารละลายจะเห็นการเปลี่ยนแปลงของสัญญาณฟลูออเรสเซนซ์เพิ่มขึ้นอย่างชัดเจน นอกจากนี้ยังพบการเพิ่มขึ้นของสัญญาณฟลูออเรสเซนซ์เล็กน้อย เมื่อเติม ATP, ADP และ GDP ตามลำดับ ในขณะที่นิวคลีโอไทด์อื่นๆ ไม่เกิดการเปลี่ยนแปลงซึ่งแสดงให้เห็นว่าเซนเซอร์ Pydpa-Zn มีความจำเพาะเจาะจงกับ PPI $\gg$ ATP > ADP > GDP แสดงดังภาพที่ 4A และค่าคงที่ในการเกิดสารประกอบเชิงซ้อน ($\log K$) ระหว่าง Pydpa-Zn กับ PPI เท่ากับ 8.83 และอัตราส่วนในการเกิดสารประกอบเชิงซ้อนระหว่าง Pydpa-Zn กับ PPI เป็น 2 ต่อ 1 ในกรณีของเซนเซอร์ Pydpa-Cu

จะพบพีคที่ 475 nm ซึ่งเป็นตำแหน่ง excimer ของไพรีน และไม่สามารถเกิดสารประกอบเชิงซ้อนกับนิวคลีโอไทด์ได้ เนื่องจาก Cu^{2+} ไม่มีออร์บิทัลว่างพอที่จะโคออร์ดิเนตกับนิวคลีโอไทด์แสดงดังภาพที่ 4B

สำหรับเซนเซอร์ Es-Zn และ Es-Cu จะเกิดการคายแสงที่ความยาวคลื่น 470 nm และ 468 nm ตามลำดับ ภาพที่ 4C แสดงการเปลี่ยนแปลงสัญญาณฟลูออเรสเซนซ์ของเซนเซอร์ Es-Zn เพิ่มขึ้นเมื่อเติมนิวคลีโอไทด์ลงในสารละลายจากการทดลองสามารถแยกนิวคลีโอไทด์ออกเป็นกลุ่มได้ดังนี้ PPI, ไตรฟอสเฟต (ATP, UTP), ไดฟอสเฟต (ADP, UDP, GDP) และมอนอฟอสเฟต (AMP, UMP, GMP, CMP) ในส่วนของเซนเซอร์ Es-Cu จะพบการเปลี่ยนแปลงของสัญญาณฟลูออเรสเซนซ์อย่างชัดเจน เมื่อเติม PPI สำหรับ ATP, UTP และ ADP และมีการเพิ่มขึ้นของสัญญาณฟลูออเรสเซนซ์เล็กน้อยแสดงดังภาพที่ 4D ค่าคงที่ในการจับ ($\log K$) ระหว่าง Es-Zn และ Es-Cu กับ PPI เท่ากับ 5.23 และ 4.64 ตามลำดับ และอัตราส่วนในการเกิดสารประกอบเชิงซ้อนระหว่าง Es-Zn และ Es-Cu กับ PPI เป็น 1 ต่อ 1

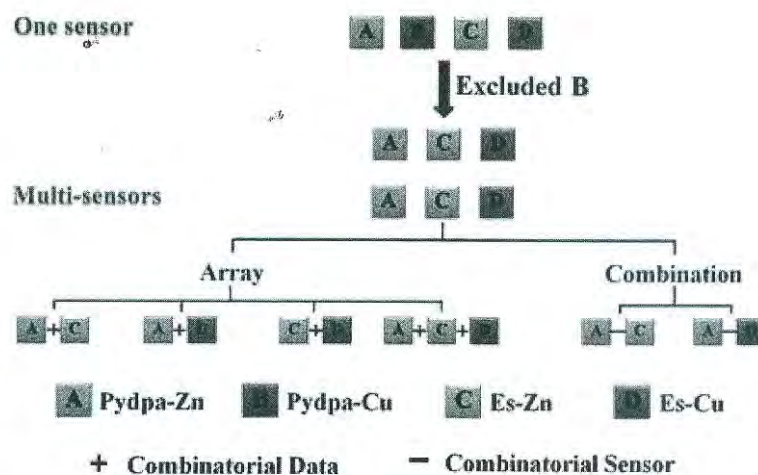




ภาพที่ 4 ฟลูออเรสเซนส์สเปกตรัมเมื่อเติมนิวคลีโอไทด์เป็น 10 เท่า ของ A) **Pydpa-Zn**, B) **Pydpa-Cu**, C) **Es-Zn** และ D) **Es-Cu**

พิจารณการตรวจวัดนิวคลีโอไทด์ด้วยเซนเซอร์ทั้งหมด (**Pydpa-Zn**, **Pydpa-Cu**, **Es-Zn** และ **Es-Cu**) พบว่าเซนเซอร์ส่วนใหญ่มีความจำเพาะเจาะจงกับ PPI ในขณะที่ **Pydpa-Cu** ไม่สามารถใช้เป็นเซนเซอร์ในการตรวจวัดนิวคลีโอไทด์ได้ เนื่องจากไม่มีการเปลี่ยนแปลงของสัญญาณฟลูออเรสเซนส์เมื่อเติมนิวคลีโอไทด์จากข้อมูลเหล่านี้เราสามารถตรวจวัดนิวคลีโอไทด์โดยแยก PPI ออกมาได้โดยใช้เทคนิคฟลูออเรสเซนส์

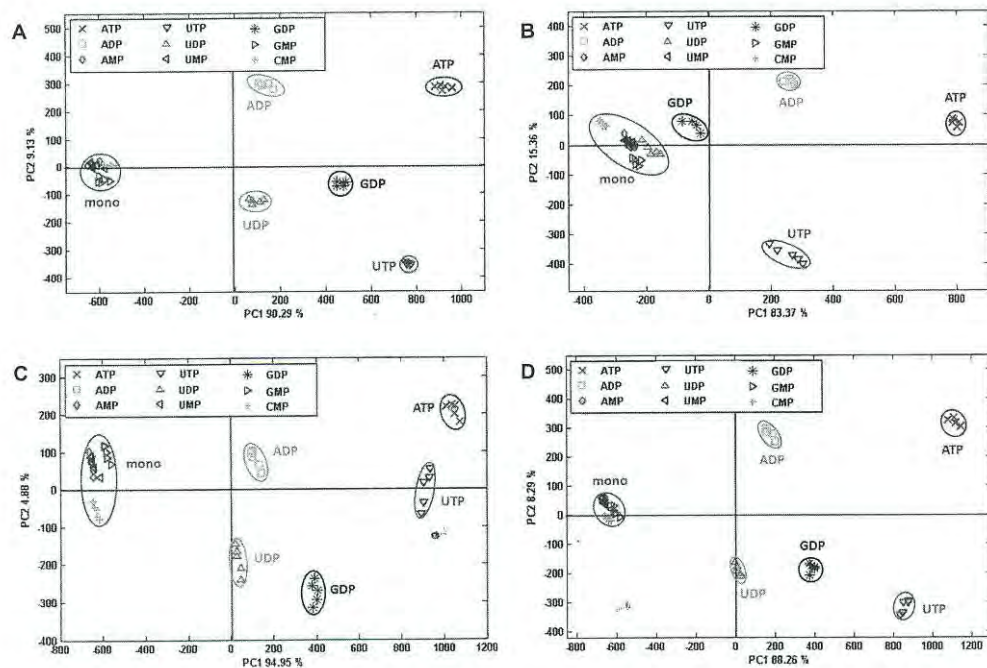
สเปกโตร สโกปี ในขณะที่นิวคลีโอไทด์ชนิดอื่นแยกได้ไม่ชัดเจน ดังนั้นจึงใช้เทคนิค Principal component analysis (PCA) เข้ามาช่วยในการจำแนกประเภทของนิวคลีโอไทด์ชนิดต่างๆ ซึ่ง PCA เป็นขั้นตอนทางคณิตศาสตร์ เพื่อใช้ในการลดจำนวนของข้อมูลในการวิเคราะห์ให้น้อยลง โดยยังคงข้อมูลเดิมทั้งหมด ซึ่งรูปแบบในการพิจารณาเป็นดังภาพที่ 5



ภาพที่ 5 ขั้นตอนในการจำแนกประเภทของนิวคลีโอไทด์ โดยใช้ Principal component analysis (PCA)

การนำหลักการทางสถิติมาประยุกต์ใช้ในการจำแนกนิวคลีโอไทด์ จะใช้ข้อมูลฟลูออเรสเซนซ์ที่ได้จากการศึกษาการเกิดสารประกอบเชิงซ้อนระหว่างเซนเซอร์ **Pydpa-Zn**, **Es-Zn** และ **Es-Cu** กับนิวคลีโอไทด์ จากการศึกษาข้างต้นพบว่าเซนเซอร์ส่วนใหญ่สามารถแยก **PPi** ออกจากนิวคลีโอไทด์อื่นๆ ได้อย่างชัดเจน แต่ไม่สามารถแยกความแตกต่างของนิวคลีโอไทด์อื่นได้ ดังนั้นจึงใช้หลักการของ PCA เข้ามาช่วยในการแยกประเภทของนิวคลีโอไทด์ โดยจะไม่นำข้อมูลของ **PPi** มาพิจารณา ภาพที่ 6 แสดง PCA score plot

ของเซนเซอร์แอเรีย **Pydpa-Zn / Es-Zn**, **Pydpa-Zn / Es-Cu**, **Es-Zn / Es-Cu** และ **Pydpa-Zn / Es-Zn / Es-Cu** จากผลของ PCA plot พบว่า **Pydpa-Zn / Es-Zn**, **Es-Zn / Es-Cu** และ **Pydpa-Zn / Es-Zn / Es-Cu** สามารถแยกนิวคลีโอไทด์ ATP, ADP, UTP, UDP และ GDP ได้อย่างชัดเจน ซึ่งจากผลของ PCA พบว่าการจำแนกประเภทนิวคลีโอไทด์ไม่จำเป็นต้องใช้เซนเซอร์ทั้งหมด เพียงแค่สองเซนเซอร์ก็สามารถแยกได้ โดยใช้เซนเซอร์แอเรีย **Pydpa-Zn / Es-Zn** หรือ **Es-Zn / Es-Cu** ดังภาพที่ 6A และ 6C



ภาพที่ 6 PCA score plot ของเซนเซอร์แอเรีย A) **Pydpa-Zn / Es-Zn**, B) **Pydpa-Zn / Es-Cu** C) **Es-Zn / Es-Cu** และ D) **Pydpa-Zn / Es-Zn / Es-Cu**

นอกจากนี้พบว่า เซนเซอร์ **Pydpa** และ **Es** ที่จับกับ Zn^{2+} และ Cu^{2+} มีความสามารถในการจับกับนิวคลีโอไทด์ได้แตกต่างกัน ดังนั้นผู้วิจัยจึงได้สร้างเซนเซอร์ใหม่ขึ้น เพื่อศึกษาอัตราในการแข่งขันการเกิดสารประกอบเชิงซ้อนกับนิวคลีโอไทด์ และใช้หลักการ PCA จำแนกประเภทนิวคลีโอไทด์

โดยนำเซนเซอร์ **Pydpa-Zn** ผสมกับ **Es-Zn** (**Pydpa-Zn+Es-Zn**) และ **Pydpa-Zn** ผสมกับ **Es-Cu** (**Pydpa-Zn+Es-Cu**)

สำหรับเซนเซอร์ **Pydpa-Zn+Es-Zn** ได้กระตุ้นที่ความยาวคลื่น 346 nm และให้สัญญาณการคายแสงที่ 383 nm และ 470 nm



Highly specific-glucose fluorescence sensing based on boronic anthraquinone derivatives via the GOx enzymatic reaction

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ABSTRACT

The boronic acid anthraquinones, **oHAQB** and **pHAQB**, have been designed, and demonstrated to serve as fluorogenic biosensors for glucose. The sensory molecule, **oHAQB**, has exhibited the specific-glucose sensing via the GOx enzymatic reaction. In this contribution, the fluorescence changes of **oHAQB** reasonably correspond to the concentration of glucose upon the conversion of boronic acid to hydroxy based sensor by H₂O₂ generated from glucose along with GOx enzymatic reaction. Our sensing ensemble was then successfully applied to determine the glucose concentration in the range of 0.08–0.42 mM. The limit of detection (LOD) of **oHAQB** for glucose detection using the GOx enzymatic probe is approximately 0.011 mM.

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1. Introduction

Diabetes is a disease, which involves abnormal blood sugar metabolism due to inadequate insulin production. Blood sugar, especially glucose, has regularly been used to diagnose diabetes for over a century. Nowadays, the capability to accurately monitor blood glucose concentrations has proven essentially for diagnosing and curing many metabolic disorders, mainly diabetes.¹ Therefore, reliable and accurate saccharide sensors are continually developed in order to search for better ones for quantitative analysis and detection of saccharides and their derivatives in physiological media. Moreover, the indispensability of glucose monitoring in the incurable disease is a critical requirement for determination of the glucose levels in nondiabetic acute care patients because high variations in blood glucose level have also been observed in non-diabetic patients as a common sign of acute illness. Patients can modify their lifestyle to better control their diabetes and can use insulin in achieving effective dosing and timing.² The important demands for continuous, accurate, and relatively noninvasive glucose sensing methods have motivated methodologies as well as the design of various sensing materials.³ For specific-glucose sensing, various analytical methods such as amperometry, potentiometry, spectrophotometry, or fluorometry are utilized for the enzymatic measurements of glucose concentration.⁴ The traditional

commercial glucose test is electrochemical, including a monitor based on the electrical wiring of glucose oxidase, which underlies the direct conversion of the concentration-dependent glucose flux to an electrical current. In this method, the generated hydrogen peroxide is oxidized under a constant working potential, and the extent of oxidation corresponds to the glucose concentration.⁵ However, it needs careful calibration because other oxidized products may be easily adsorbed onto the metal electrode surfaces, causing a decrease in activity of the electrode surface and the sensitivity will dramatically decrease.⁶

The development of specific sensors for biochemically relevant analytes via chemical reaction has high impact and challenging. Fluorescence sensors with preferential binding to glucose have been developed in the past decade. The obvious advantages of a fluorescence sensor are the extreme sensitivity and lack of damage to the host system. The methodology involves the incorporation of synthetic based receptors, such as boronic acid. Boronic acid based sensors are the best candidate for saccharide probes due to the covalent binding properties of boronic acid with the diol group of sugars in water as widely reported for the last decade.⁷ Moreover, most boronic acid derivatives based fluorophores showed the fluorescence emission band at a relatively long wavelength, which is an important role in detecting glucose in blood or serum samples. However, the disadvantages of some boronic acid based fluorescence sensor are the selectivity and a high pK_a values, which is not appropriate for physiological pH.⁸ Moreover, a number of publications have reported the glucose-specific

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fluorescence changes with the interference of other saccharides especially fructose and ribose.⁹ A few reports exist regarding the selective fluorogenic boronic acid for glucose sensing.¹⁰ As the basic knowledge of boronic acid, aryl boronic acid and boronic ester react selectively with H_2O_2 to produce a phenolic compound.¹¹ To overcome the disadvantage of using a boronic-based fluorescence sensor for selective detection of glucose, we took an effort to combine the basic knowledge of the specific-glucose sensing by GOx enzymatic reaction with the new boronic-based anthraquinone for fluorescence changes. Our proposition for the specific-glucose fluorescence sensor via the GOx enzymatic reaction is schematically illustrated in Fig. 1.

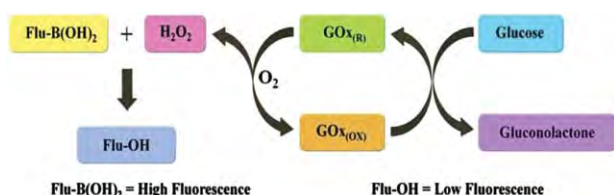


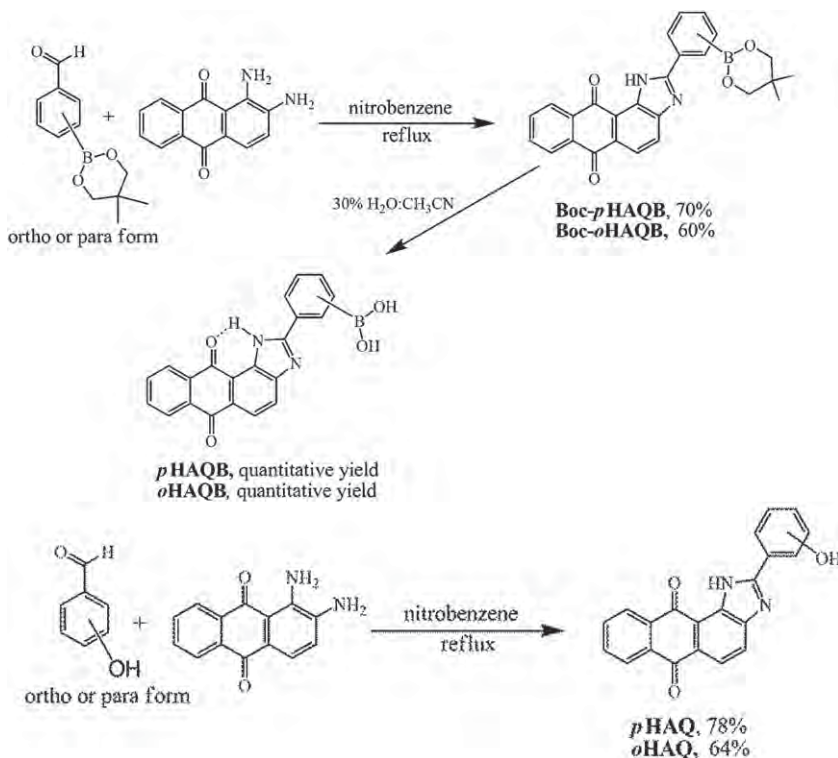
Fig. 1. Schematic illustration of the GOx enzymatic mechanism for glucose detection using boronic-based fluorescence sensors.

directly. Herein, new anthraquinone based fluorosensors, *o*HAQB and *p*HAQB containing boronic acid as the enzymatic probe for glucose has been designed and synthesized. The sensing affinity for glucose was investigated by fluorescence spectrophotometry. Among both sensors, *o*HAQB permits the selective detection of glucose upon the GOx enzymatic reaction with a detection limit of $11.4 \mu\text{M}$ and average % recovery of 104, which is indicative of powerful glucose sensing.

2. Results and discussion

2.1. Synthesis and physical properties of sensors *p*HAQB and *o*HAQB

Synthetic methodologies for the new probes, *p*HAQB and *o*HAQB are shown in Scheme 1. The heterocyclic precursors, Boc-*p*HAQB and Boc-*o*HAQB were achieved from oxidative condensation between diamino anthraquinone and formyl phenylboronated ester with 70% and 60% yields, respectively. The characterization of all sensors was carried out using ^1H as well as ^{13}C NMR and HRMS. For ^1H NMR spectra, the appearance of a broad singlet signal at around 13.76 and 12.97 ppm for *p*HAQB and *o*HAQB, respectively, supports the formation of intramolecular hydrogen bonding between the NH of the imidazole ring and the neighboring quinone carbonyl group.¹²



Scheme 1. Synthesis of sensory molecules, *p*HAQB, *o*HAQB, *p*HAQ and *o*HAQ.

For our hypothesis, the H_2O_2 generated by the reaction between glucose and glucose oxidase would convert the boronic acid to the phenol based fluorescence sensor and the fluorescence change would be related to the concentration of glucose. Although, most organic fluorophores can exhibit fluorescence quenching by H_2O_2 , we designed the organic fluorophore containing boronic acid for specific detection of glucose upon the GOx enzymatic reaction without the interference of glucose binding to the boronic acid

Sensors *o*HAQB and *p*HAQB in 10% DMSO with 0.1 M HEPES buffer at pH 7.4 showed a strong fluorescence intensity at 609 and 589 nm, respectively, with quantum yields of 0.322 and 0.231 with respect to a typical quinone compound as shown in Fig. 2. As expected, fluorescence responses of *p*HAQ and *o*HAQ consisting of hydroxy groups were relatively low in aqueous media and consequently their quantum yields could not be determined.

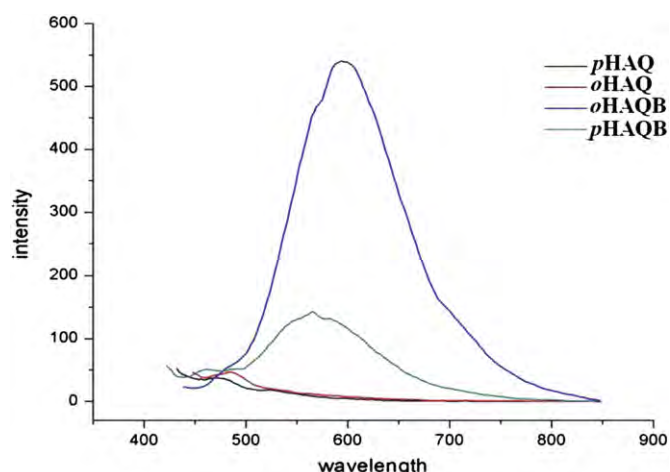


Fig. 2. Emission spectra of *pHAQB*, *oHAQB*, *pHAQ* and *oHAQ* in 10% DMSO with 0.1 M HEPES buffer at pH 7.4.

These positive preliminary results encouraged us to investigate the boronic acid based sensors as a glucose sensing under the enzymatic mechanism mentioned above.

To study the binding properties between sensors *pHAQB* and *oHAQB* toward glucose without glucose oxidase, the fluorescence titration curves exhibited large changes of fluorescence quenching for *pHAQB* but the fluorescence quenching of *oHAQB* in the presence of glucose displayed insignificant change as shown in Fig. 3. As a result of steric hindrance of boronic binding site of *oHAQB* toward glucose, *pHAQB* preferred to bind glucose over *oHAQB*. Moreover, the binding constants of *oHAQB* and glucose cannot be determined.

We, therefore, reasoned that *oHAQB* was a better candidate for enzymatic activation as it would be no competition from direct binding.

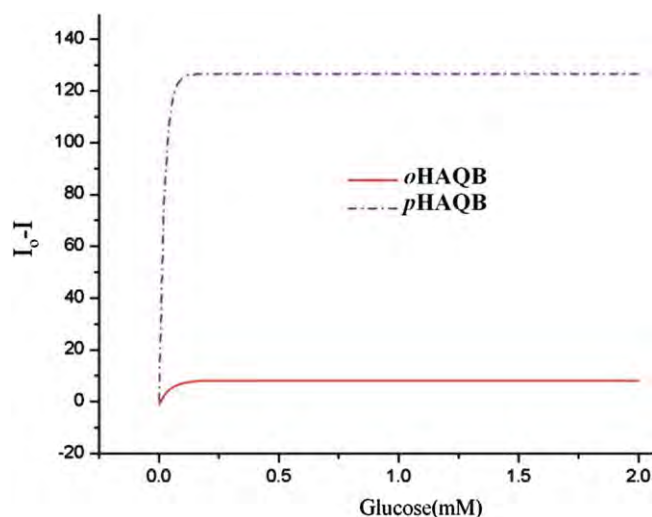


Fig. 3. Fluorescence titration curves of 20 μ M *oHAQB* and *pHAQB* at 590 and 560 nm, respectively, with glucose in 100 mM HEPES buffer (pH 7.4).

2.2. Enzymatic detection of glucose using *oHAQB*

Prior to the use of enzymatic mechanism of GOx and glucose, the optimized conditions of the system were investigated in terms of amount of GOx unit, temperature and reaction time. From the fluorescence results as shown in ESI, the appropriate condition for

the high efficiency of enzymatic probe was performed by six units of GOx at a temperature of 37 °C and reaction time of 60 min. These appropriate conditions were utilized for all manipulations related to the enzymatic reaction of GOx.

From the literature review¹³ reporting the binding ability between GOx and boronic acid, we thus tested the binding ability of *oHAQB* and GOx by fluorescence spectrophotometry. We found unchanged emission spectra of *oHAQB* in the presence of GOx as shown in Fig. S5. This indicated unbound *oHAQB* with GOx suggesting no influence of the complexation of *oHAQB* and GOx toward emission spectral changes. Hence, we have focused on studying the use of *oHAQB* for detection of glucose. Hypothetically, H₂O₂ generated from the reaction of glucose and glucose oxidase would convert the boronic acid based sensor to the hydroxy group and subsequently induced the fluorescent quenching of anthraquinone derivatives. To confirm this hypothesis, the product of the boronate-based fluorescent probe (*oHAQB*) hydrolyzed by H₂O₂ generated from the reaction of the glucose and GOx was investigated by ESI-MS (shown in ESI), which showed the peak of *m/z* 340.08 corresponding to the structure of *oHAQ* (the hydrolyzed boronate-based *oHAQB*).

We first examined the comparison of fluorescence changes of *oHAQB* upon direct addition of H₂O₂ and the addition of glucose along with GOx as shown in Fig. 4.

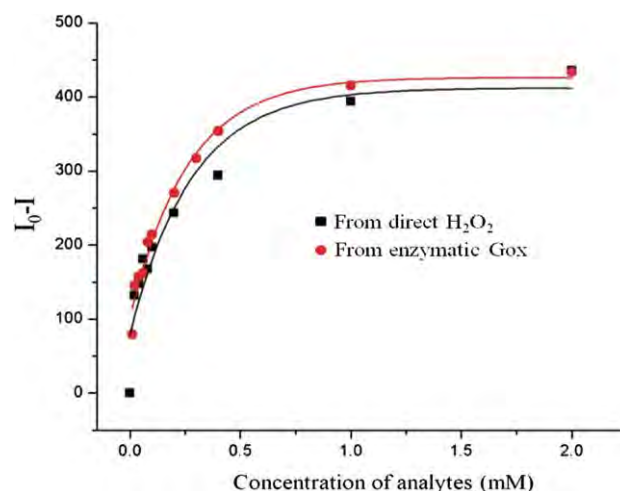


Fig. 4. Fluorescence titration curves (at 609 nm) of 20 μ M *oHAQB* upon addition of direct H₂O₂ and addition of glucose and GOx in 100 mM HEPES buffer (pH 7.4).

As anticipated, the fluorescence responses of *oHAQB* in the presence of H₂O₂ by direct addition, and H₂O₂ generated by the reaction of glucose and glucose oxidase were consistent and almost overlapped. The small difference of fluorescence responses from both systems is acceptable. In this contribution, monitoring based on the fluorescent changes of *oHAQB* stemmed from the direct conversion of the glucose by glucose oxidase corresponding to the schematic illustration in Fig. 1. Therefore, we envisaged the use of *oHAQB* as the enzymatic GOx probe for glucose. To prove the selectivity of *oHAQB* for specific-glucose sensing, the effects of interfering saccharides (2.0 mM) including galactose, fructose, sucrose and maltose were evaluated in the determination of 0.2 mM of glucose with and without GOx in 100 mM HEPES buffer (pH 7.4).

Regarding the fluorescence responses of *oHAQB* shown in Fig. 5, poor responses were readily observed for galactose and fructose with fluorescent enhancement as well as sucrose and maltose with fluorescence quenching. On the other hand, the observation of a large fluorescent enhancement of *oHAQB* in the presence of

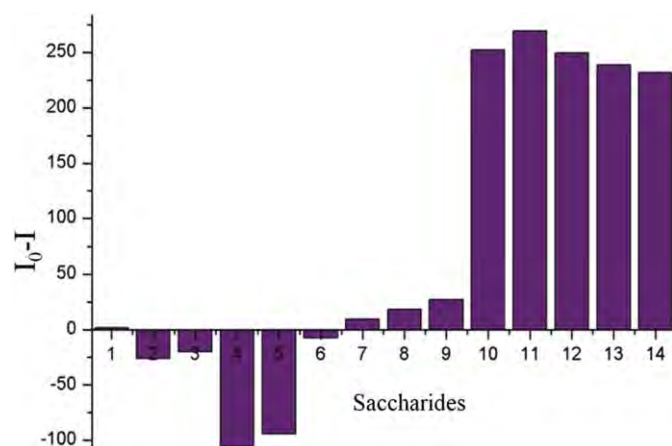


Fig. 5. Fluorescent responses of **oHAQB** (20 μ M) in the presence of 0.2 mM glucose [1] or 2 mM of the competitive saccharides: galactose [2], galactose+GOx [3], fructose [4], fructose+GOx [5], sucrose [6], sucrose+GOx [7], maltose [8], maltose+GOx [9], glucose+GOx [10] and the mixture of glu+GOx with galactose [11], fructose [12], sucrose [13], and maltose [14].

glucose along with GOx as shown in Fig. 5 could indicate a highly specific recognition for glucose. Interestingly, other saccharides did not affect the glucose detection under the enzymatic GOx mechanism.

To verify the sensing application of the boronic anthraquinone based sensors (**oHAQB**), the fluorescence titration between **oHAQB** and glucose in the presence of glucose oxidase was investigated in the optimum conditions. As depicted in Fig. 6, the emission band at 609 nm of **oHAQB** was gradually decreased upon the increment of glucose. To normalize the fluorescence titration of enzymatic probe system of **oHAQB**, the calibration curve was plotted between I_0-I and the glucose concentration (shown in inset of Fig. 6). The fluorescence response was linear in the range of 0.08–0.42 mM with an acceptable correlation coefficient (R^2) of 0.99082. Therefore, the range of concentration for glucose sensing by the GOx enzymatic probe system is 0.08–0.42 mM. The limit of detection (LOD) of **oHAQB** for glucose detection using GOx enzymatic probe is approximately 0.0114 mM or 11.4 μ M.

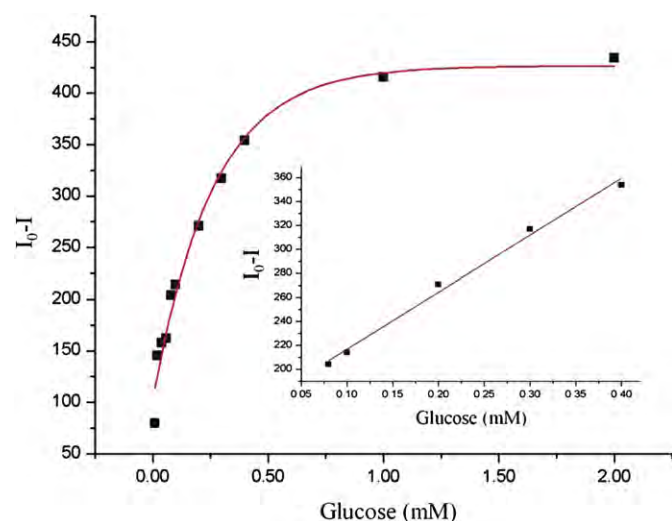


Fig. 6. Fluorescence titration curve of 20 μ M **oHAQB** and six units of glucose oxidase titrated with glucose in 10% DMSO with 0.1 M HEPES buffer at pH 7.4. The excitation wavelength was 415 nm. The reaction was performed at 37 $^{\circ}$ C and 1 h and inset: linear calibration curve of **oHAQB** in the same condition.

In analytical applications using this spectrofluorometric method, **oHAQB** was applied to detect glucose in drinking water. The % recoveries (shown in Table 1) of the spike samples of **oHAQB** were in the range of 91–116 and the average % recovery of 104, which is acceptable for analytical application.

Table 1

Analysis of glucose using **oHAQB** with the enzymatic reaction of GOx in drinking water

Added glucose (mM)	Found (mM)	% Recovery
0.05	0.51	102
0.10	0.091	91
0.15	0.175	116
0.20	0.216	108
0.30	0.313	104

3. Conclusions

A fluorescence sensory molecule, **oHAQB**, has been successfully synthesized, and its glucose sensing capability has been developed under enzymatic mechanism of GOx. All corresponding results are consistent with the hypothesis that H_2O_2 generated from the enzymatic reaction of glucose and GOx showed high affinity to hydrolyze the boronic acid group to the hydroxy moiety with the proportional fluorescence changes of the sensor, **oHAQB**. To the best of our knowledge, this is the first report of the use of boronic-based fluorescence biosensor to detect glucose under the GOx enzymatic reaction. Consequently, **oHAQB** serves as an excellent biosensor for glucose sensing with high selectivity. The proposed mechanism highlighted the benefits of further use of the simple fluorescence sensor as the GOx enzymatic probe for detection of glucose in real-time analysis in the field of medical diagnosis and food safety.

4. Experimental section

4.1. Materials and general methods

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Mercury Plus 400 NMR spectrometer. All chemical shifts were reported in parts per million (ppm) using the residual proton or carbon signal in deuterated solvents as internal references. MALDI-TOF mass spectra were carried out on Bruker Daltonics MALDI-TOF using 2-cyano-4-hydroxy cinnamic acid (CCA) as matrix.

All materials and solvents chemicals were purchased from Aldrich, Fluka and Merck as standard analytical grade and were used without further purification. Commercial grade solvents such as dichloromethane, ethyl acetate, hexane, and methanol were purified by distillation. Anhydrous solvents such as dichloromethane were dried over CaH_2 and freshly distillation under nitrogen atmosphere. Column chromatography was carried out on silica gel (Kieselgel 60, 0.063–0.200 mm, Merck). Thin layer chromatography (TLC) was performed on silica gel plates (Kieselgel 60, F₂₅₄, 1 mm). Compounds on TLC plates were detected by the UV-light.

For UV/vis and Fluorescence analysis, compounds were dissolved in dimethyl sulfoxide (DMSO, fluorometric grade, Dojindo) to obtain 1 mM stock solutions. These stock solutions were diluted with buffer as specified in the figure legends to the desired concentration. For determination of the quantum efficiency of fluorescence (Φ_f), were calculated using the integrated emission intensity of quinine sulfate as standard. All absorption spectra were obtained with Hewlett Packard 8452A Diode Array Spectrometer.

All fluorescence spectra were obtained with Varian Cary Eclipse Fluorescence Spectrophotometer. The boronate esters were synthesized according to the previously published procedure.¹⁴

4.2. Synthesis

Preparation of 2-(R-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl)phenyl)-1H-anthra[1,2-d]imidazole-6,11-dione (R=2, 4). Into a two-neck round bottom flask equipped with a magnetic bar, the corresponding formyl phenylboronate ester (1 mmol) in nitrobenzene (25 mL) was added dropwise to a solution of 1,2-diamino-1,4-anthraquinone (1 mmol) in nitrobenzene (75 mL).

The reaction mixture was slowly heated to 150 °C for 1 day under nitrogen atmosphere. The solution was cooled to room temperature and then the precipitate slowly formed. The precipitate was filtered and washed with diethylether to give a brown solid of the protecting products (**Boc-oHAQB**=45%, **Boc-pHAQB**=60%). ¹H NMR (DMSO, 400 MHz) **Boc-oHAQB**, δ (ppm): 13.56 (s, 1H), 8.41 (d, 1H, $J=6.4$ Hz), 8.25–8.13 (m, 4H), 7.94–7.92 (m, 2H), 7.57–7.50 (m, 3H), 3.70 (s, 4H), 1.07 (s, 6H). **Boc-pHAQB**, δ (ppm): 10.18 (s, 1H), 8.40 (t, 1H, $J=8.2$ Hz), 8.29–8.21 (m, 4H), 8.13–8.05 (m, 2H), 7.97–7.93 (m, 3H), 3.78 (s, 2H), 3.11 (s, 2H), 0.97 (s, 3H) 0.72 (s, 3H).

Preparation of R-(6,11-dioxo-6,11-dihydro-1H-anthra[1,2-d]imidazol-2-yl)phenylboronic acid (R=2: oHAQB, 4: pHAQB). Protecting groups of 2-(R-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl)phenyl)-1H-anthra[1,2-d]imidazole-6,11-dione (R=2:2o, 4: 2p) (1 mmol) were removed by refluxing in 30 mL of 30% H₂O:CH₃CN. The yellow solid of product **oHAQB** and brown solid of product **pHAQB** was filtered off and washed with diethylether to provide the desired product in a quantitative yield.

Characterization data for oHAQB: Melting point 350–354 °C; ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 13.76 (s, 1H), 8.26–8.21 (m, 2H), 8.14 (d, 2H, $J=6.8$ Hz), 7.99 (d, 2H, $J=6.0$ Hz), 7.94–7.92 (m, 1H), 7.75 (d, 1H, $J=8.4$ Hz), 7.68 (d, 2H, $J=6.8$ Hz). ¹³C NMR (100.6 MHz, DMSO-*d*₆) δ =182.36, 182.00, 181.50, 159.60, 148.58, 133.60, 132.59, 132.33, 131.91, 129.68, 129.23, 128.49, 128.04, 126.84, 126.66, 126.23, 126.12, 124.74, 121.60, 120.93, 118.58. IR spectrum (KBr, (cm⁻¹)): 3421 (N–H (2°-amines) stretching, wk), (2935 (C=C, stretching) 1657 (C=O stretching, str), 1584, 1490 (C=C, stretching, str), 1300 (C–N) stretching). HRESI-MS calculated for C₂₁H₁₃N₂O₄: 369.12 [M], observed: 387.09 [M+H₂O]=419.123.

Characterization data for pHAQB: Melting point 366–367 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ =12.97 (s, 1H), 8.34 (d, 1H, $J=7.6$ Hz), 8.26 (s, 2H), 8.06–8.25 (m, 2H), 7.96 (m, 4H), 7.80 (m, 2H); ¹³C NMR (100.6 MHz, DMSO-*d*₆) δ =183.37, 182.56, 158.60, 137.53, 134.76, 134.54, 133.36, 133.23, 133.160, 128.33, 127.47, 127.09, 126.50, 125.27, 121.33, 118.91; IR spectrum (KBr, (cm⁻¹)): 3415 (N–H (2°-amines) stretching, wk), (1658 (C=O stretching, str), 1523, 1470 (C=C, stretching, str), 1298 (C–N) stretching, med), 723 (C–H bending, str); Elemental analysis: Anal. Calcd for: C₂₁H₁₃N₂O₄: C, 68.51; H, 3.56; N, 7.61; found: C, 68.57; H, 3.56; N, 7.62. MALDI-TOF: m/z for (M+3H)⁺=382.11.

4.3. Glucose sensing studies

4.3.1. The complexation studies of the boronic anthraquinone based sensors with glucose using fluorescence titration experiments. Typically, the sensors were prepared in spectroscopic grade DMSO at concentrations of 2.00×10^{-4} mol/L as a stock solution. The stock solutions of glucose were prepared at concentrations of 6.00×10^{-3} mol/L in 0.1 M HEPES buffer pH 7.4. In a 5.0 mL beaker, 0.30 mL of the stock solution of sensor was mixed with the portions of stock solution of glucose to give final concentration of glucose. After volumes were adjusted to 3.00 mL with 0.1 M HEPES buffer (pH 7.4) to give a final concentration of sensors at 2.00×10^{-5} mol/L

in 10% DMSO aqueous solution, the mixtures was stirred at room temperature for 10 min and placed in a 100.0 mm width quartz cell and then fluorescence spectra were recorded at 25 °C.

4.3.2. Fluorescence measurement of boronic anthraquinone based sensors (oHAQB) in optimum condition of the enzymatic probe system for glucose detection by glucose oxidase. Typically, the sensors were prepared in spectroscopic grade DMSO at concentrations of 2.00×10^{-4} mol/L as a stock solution. The stock solutions of glucose were prepared at concentrations of 6.00×10^{-3} mol/L in 0.1 M HEPES buffer pH 7.4. In a 10.0 mL vial, 0.30 mL of the stock solution of sensor was mixed with the portions of stock solution of glucose to give final concentration of glucose. After volumes were adjusted to 3.00 mL with 0.1 M HEPES buffer pH 7.4 to give a final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution, then six units of glucose oxidase were added to the solution mixture of the sensor and the glucose. The mixtures was stirred at 37 °C for 60 min and placed in a 100.0 mm width quartz cell and then, the fluorescence spectra were recorded at 25 °C. For kinetic studies, the fluorescence spectra were recorded at 25 °C every 5 min.

4.3.3. Competitive studies of other saccharides. In a 10.0 mL vial, 0.30 mL of 2.00×10^{-4} mol/L of a sensor in spectroscopic DMSO was mixed with 100 μ L of 6.00×10^{-2} mol/L of stock solutions including D-glucose, D-galactose, D-fructose, D-sucrose and D-maltose in 0.1 M HEPES buffer pH 7.4 and all solutions was adjusted to 3.00 mL to give final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution. Then six units of glucose oxidase were added to the solution mixture of the sensor and the saccharides. The mixture solution was stirred at 37 °C for 60 min and placed in a 100.0 mm width quartz cell and then fluorescence spectra were recorded at room temperature.

4.3.4. Glucose detection of drinking water sample. The commercially drinking water sample was used for volume adjustment with 0.1 M HEPES buffer pH 7.4 to give a final concentration of 10% DMSO aqueous solution of sample. The glucose stock solution was spiked to the mixture solution of **oHAQB** to give the final concentration of 0.05–0.3 mM of glucose and 2.00×10^{-4} M of **oHAQB** and then 6 units of glucose oxidase was added to the solution mixture of the sensor and the glucose. After stirring the mixture solution at temperature of 37 °C for 60 min, the sample mixture was then recorded at the room temperature.

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Supplementary data

¹H NMR spectrum of **oHAQB** and **pHAQB**. Fluorescence spectra and Mass spectroscopy. Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.tet.2012.08.037>.

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