

DEVELOPMENT OF NEAR-INFRARED PHOTSENSITIZER
NANOPARTICLES FOR ENHANCED PHOTODYNAMIC CANCER
THERAPY



PRAPASSARA MUANGSOPA

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การพัฒนาอนุภาคนาโนของสารไวแสงในช่วงอินฟราเรดช่วงคลื่นสั้นสำหรับ
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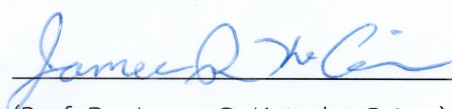


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DEVELOPMENT OF NEAR-INFRARED PHOTSENSITIZER NANOPARTICLES
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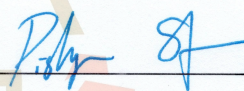
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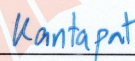
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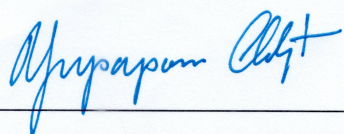
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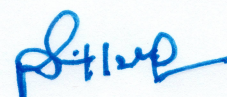
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Member



(Assoc. Prof. Dr. Yupaporn Ruksakulpiwat)

Vice Rector for Academic Affairs
and Quality Assurance



(Prof. Dr. Santi Maensiri)

Dean of Institute of Science

ประภัสรา ม่วงโสภา : การพัฒนานอนุภาคนาโนของสารไวแสงในช่วงอินฟราเรดช่วงคลื่นสั้น สำหรับเพิ่มประสิทธิภาพการรักษามะเร็งโดยใช้แสงเป็นตัวกระตุ้น (DEVELOPMENT OF NEAR-INFRARED PHOTOSENSITIZER NANOPARTICLES FOR ENHANCED PHOTODYNAMIC CANCER THERAPY) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.อัญญาณี คำแก้ว, 101 หน้า.

คำสำคัญ : เอซาบอดิปี; เฮปตะเมธินไฮยานีน; สารเรืองแสงในช่วงอินฟราเรดช่วงคลื่นสั้น; การบำบัดด้วยการกระตุ้นด้วยแสง; เทคโนโลยีนาโน

การรักษามะเร็งโดยใช้แสงเป็นตัวกระตุ้นถือเป็นวิธีการรักษาที่มีแนวโน้มดี เนื่องจากไม่รุกรานร่างกายและมีความจำเพาะสูง ในงานวิจัยนี้ คณะผู้วิจัยเสนอการพัฒนานอนุภาคนาโนสำหรับรักษาโรคมะเร็งที่ผสมผสานการใช้ในการรักษาและการวินิจฉัยในตัวเดียว อนุภาคนาโนอินทรีถูกพัฒนาจากสารไวแสงสองชนิดที่แตกต่างกันได้แก่ 1) สารเรืองแสงจำพวกอะมิโนเฮปตะเมธินไฮยานีน และ 2) เอซาบอดิปี ซึ่งทั้งสองชนิดเหมาะสำหรับการพัฒนาโพรบเรืองแสงสำหรับการถ่ายภาพมะเร็งด้วยแสงในช่วงอินฟราเรดใกล้และการบำบัดด้วยแสง โดยกำหนดเป้าหมายไปที่เซลล์มะเร็งผ่านอแฟกต์การซึมผ่านและการกักเก็บที่เพิ่มขึ้น

สำหรับอนุภาคนาโนจากสารเรืองแสงไฮยานีน ได้มีการพัฒนาระบบนาโนที่ปราศจากตัวพาสำหรับการถ่ายภาพด้วยแสงในช่วงใกล้อินฟราเรดและการบำบัดด้วยแสง ร่วมกับเคมีบำบัด โดยการประกอบสารไวแสงและสารเคมีบำบัดเข้าด้วยกันอย่างง่าย เราใช้ไฮยานีนที่เติมไอโอดีน (I_2 -IR783) เป็นตัวแทนของสารไวแสง และสร้างอนุภาคขนาดนาโนกับเมโทเทรกเซท (MTX) ซึ่งเป็นยาเคมีบำบัด โดยใช้วิธีการตกตะกอนนาโน โดยอนุภาคนาโนที่ได้ (I_2 -IR783/MTX@NPs) มีขนาดเฉลี่ย 240.6 ± 2.5 นาโนเมตร อนุภาคนาโนที่เตรียมได้นี้แสดงให้เห็นการแทรกซึมอย่างมีนัยสำคัญเข้าไปในเซลล์มะเร็งเต้านมของหนู และแสดงผลการต่อต้านมะเร็งร่วมกันเมื่อถูกกระตุ้นด้วยแสงที่มีความยาวคลื่น 805 นาโนเมตร หลังจากได้รับแสงในช่วงอินฟราเรดช่วงคลื่นสั้นประสิทธิภาพของการบำบัดแบบไดนามิกของระบบนาโนได้รับการพิสูจน์โดยการตรวจจับ reactive oxygen species (ROS) ภายในเซลล์และการทดสอบความมีชีวิต/ความเป็นพิษต่อเซลล์ นอกจากนี้ยังมีการใช้การตรวจด้วยเครื่อง optical coherence tomography (OCT) เป็นวิธีทางเลือกในการติดตามการตอบสนองต่อการรักษา ดังนั้น I_2 -IR783/MTX@NPs จึงแสดงให้เห็นถึงศักยภาพที่สำคัญสำหรับการประยุกต์ใช้ในการรักษามะเร็งเต้านมด้วยเคมีบำบัดควบคู่กับการบำบัดด้วยแสงเป็นตัวกระตุ้น

อนุภาคนาโนอินทรีอีกชนิดหนึ่งถูกพัฒนาขึ้นโดยใช้สารไวแสงเอซาบอดิปี เราได้นำอนุพันธ์ของเอซาบอดิปีที่ปราศจากอะตอมหนัก (AB-OH) ซึ่งมีโครงสร้าง แบบผู้ให้-ผู้รับ-ผู้ให้ ร่วมกับโพลีเอ

ทีลีนไกลคอล-ซัคซินิกแอซิดโพลีเมอร์ (mPEG-SA) เมื่อประกอบตัวเองเป็นอนุภาคนาโนแล้ว AB-OH@mPEG-SA แสดงให้เห็นถึงความสามารถในการสร้าง ROS และความร้อนระดับปานกลางได้ เมื่อถูกกระตุ้นด้วยแสงช่วงคลื่นสั้น (660 นาโนเมตร) การถ่ายภาพแบบคอนโฟคอลเปิดเผยให้เห็นว่า วัสดุนาโนถูกนำเข้าสู่เซลล์และสะสมที่เมะเร็งได้อย่างมีประสิทธิภาพมากกว่า AB-OH นอกจากนี้ อนุภาคนาโน AB-OH@mPEG-SA ยังแสดงฤทธิ์เป็นพิษต่อเซลล์ที่เกิดจากการถูกกระตุ้นด้วยแสงในเซลล์มะเร็งเต้านม ในขณะที่เซลล์ที่ไม่ได้รับการฉายรังสียังคงมีชีวิตอยู่ได้อย่างสมบูรณ์ นอกจากนี้ อนุภาคนาโนที่เตรียมได้ยังแสดงให้เห็นกิจกรรมต่อต้านมะเร็งที่เหนือกว่าเมื่อเทียบกับสารประกอบ AB-OH การศึกษานี้เน้นย้ำถึงศักยภาพของอนุภาคนาโน AB-OH@mPEG-SA ซึ่งแสดงให้เห็นถึง ประสิทธิภาพของการบำบัดมะเร็งแบบโฟโตไดนามิก ร่วมกับการบำบัดด้วยความร้อน (ในระดับปานกลาง) ที่มีแนวโน้มที่ดีในฐานะของการเป็นทางเลือกในการวินิจฉัยและรักษามะเร็ง



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ปีการศึกษา 2567

ลายมือชื่อนักศึกษา ประภัสรา ม่วงโสภา

ลายมือชื่ออาจารย์ที่ปรึกษา Dr.

PRAPASSARA MUANGSOPA : DEVELOPMENT OF NEAR-INFRARED PHOTSENSITIZER
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ADVISOR : ASSOC. PROF. ANYANEE KAMKAEW, Ph.D. 101 PP.

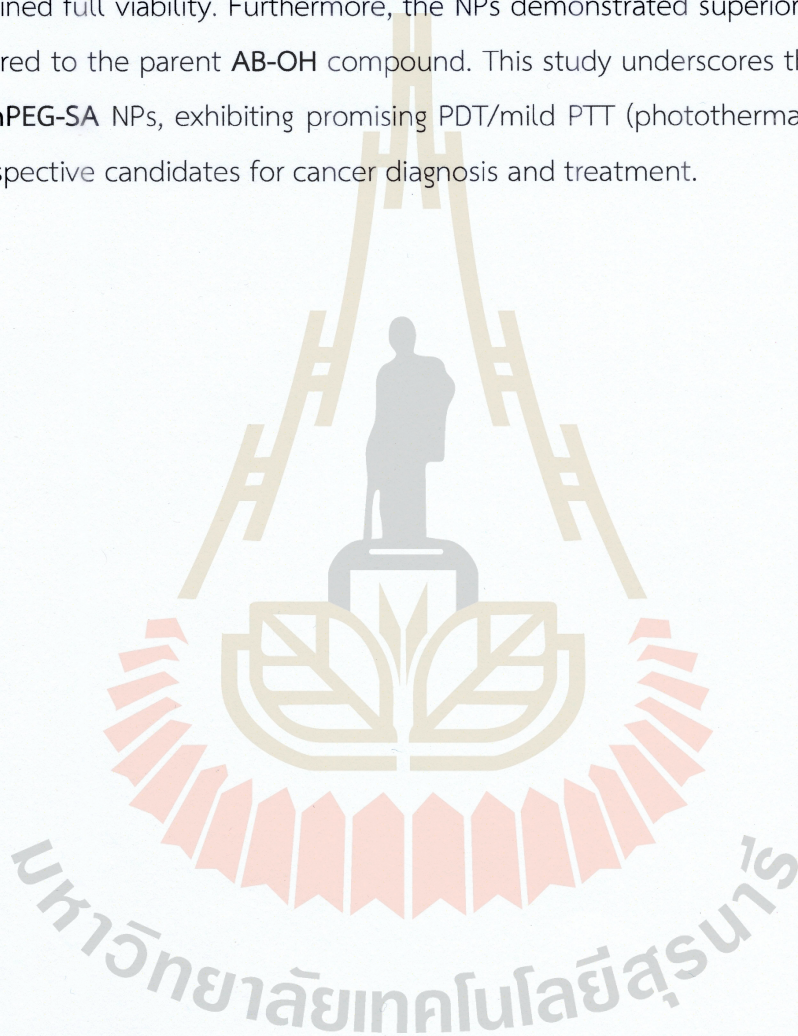
Keywords: aza-BODIPY; heptamethine cyanine; near-infrared photosensitizer;
photodynamic therapy; nanotechnology

Photodynamic therapy (PDT) stands out as a promising modality for cancer treatment due to its non-invasive nature and high specificity. In this work, we proposed the development of theranostics nanomedicines that combine therapeutic and diagnostic capabilities into a single compound. We present organic nanoparticles derived from two distinct photosensitizers (PSs); 1) a heptamethine cyanine dye and 2) an Aza-BODIPY dye, which are suitable for the development of fluorescent probes for near-infrared (NIR) fluorescence imaging and PDT, targeting cancer cells via the enhanced permeability and retention (EPR).

For cyanine-based nanoparticles (NPs), a carrier-free nanosystem for NIR imaging and PDT combined with chemotherapy was developed through the simple self-assembly of a dye and a chemotherapeutic agent. We used iodinated cyanine dye (**I₂-IR783**) as a PDT/NIR agent and formed nanoparticles with methotrexate (MTX), a chemotherapeutic drug, using a modified nanoprecipitation method. The resulting nanoparticles (**I₂-IR783/MTX@NPs**) exhibited an average size of 240.6 ± 2.5 nm. These nanoparticles demonstrated significant internalization into 4T1 murine breast cancer cells and showed a synergistic anticancer effect upon 805 nm LED lamp irradiation. After NIR light exposure, the PDT efficacy of the nanosystem in a physiological environment was validated through the detection of intracellular reactive oxygen species and a live/dead viability/cytotoxicity assay. Additionally, optical coherence tomography (OCT) was employed as an alternative method to monitor the therapeutic response. Thus, **I₂-IR783/MTX@NPs** exhibit significant potential for theranostic applications in breast cancer PDT-chemotherapy.

Another organic NP is based on Aza-BODIPY dye. We introduced a heavy-atom-free aza-BODIPY (**AB-OH**) compound featuring a donor-acceptor-donor (D-A-D) structure, coupled with poly(ethylene glycol)-succinic acid polymer (mPEG-SA). Upon self-assembly

into nanoparticles, **AB-OH@mPEG-SA** demonstrated the ability to generate reactive oxygen species (ROS) and heat when exposed to 660 nm red LED lamp irradiation. Confocal imaging revealed that the nanomaterials can be internalized and accumulated at the tumor site more effectively than the free dye. In addition, **AB-OH@mPEG-SA** displayed light-induced cytotoxicity in 4T1 cells, whereas the non-irradiated cells maintained full viability. Furthermore, the NPs demonstrated superior anticancer activity compared to the parent **AB-OH** compound. This study underscores the potential of **AB-OH@mPEG-SA** NPs, exhibiting promising PDT/mild PTT (photothermal therapy) efficacy, as prospective candidates for cancer diagnosis and treatment.



School of Chemistry
Academic Year 2024

Student's Signature PRAPASSARA MUANGSOPA
Advisor's Signature 

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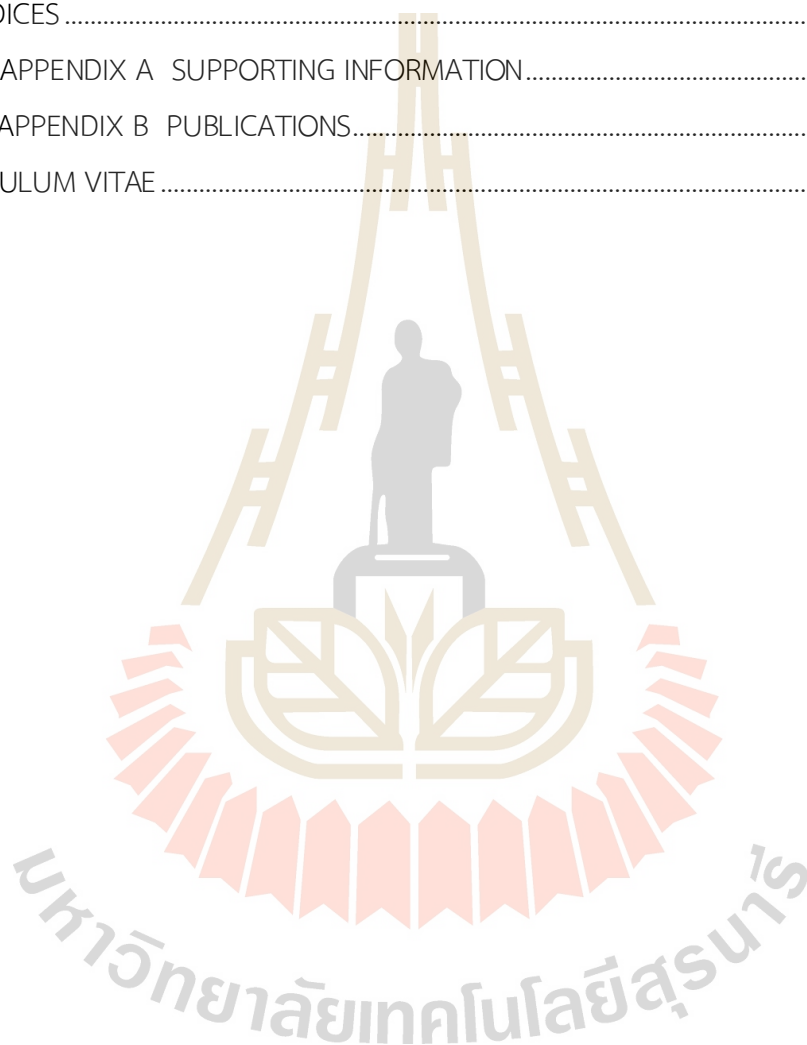
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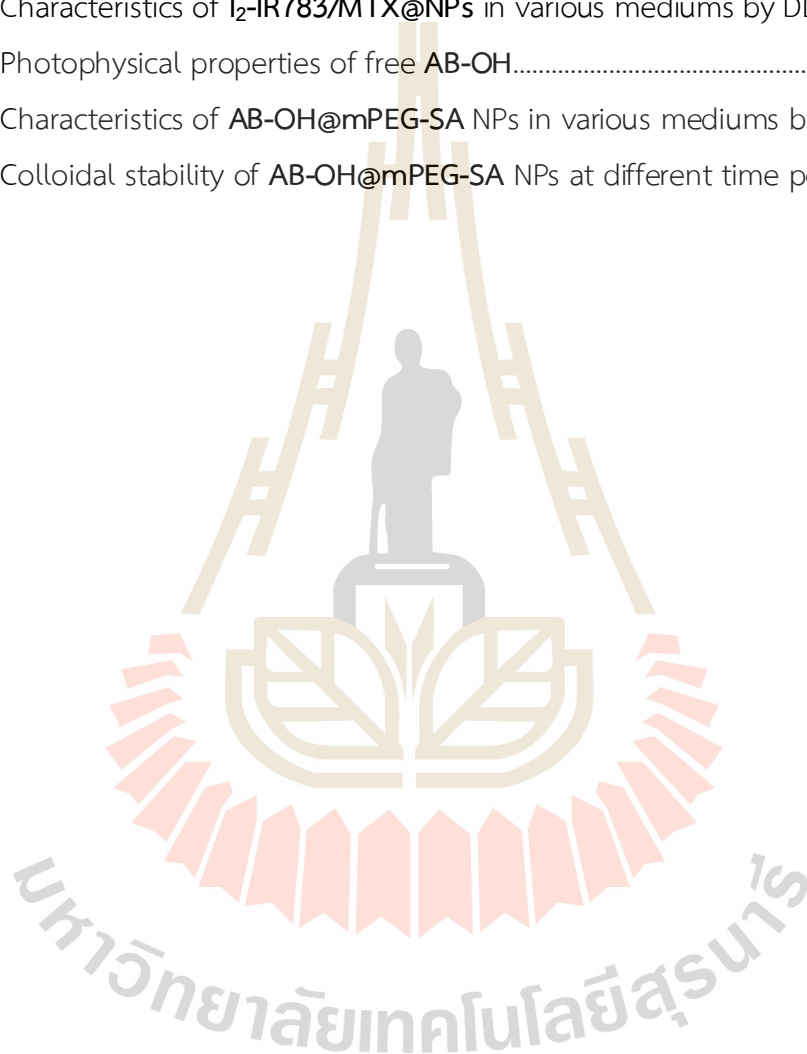
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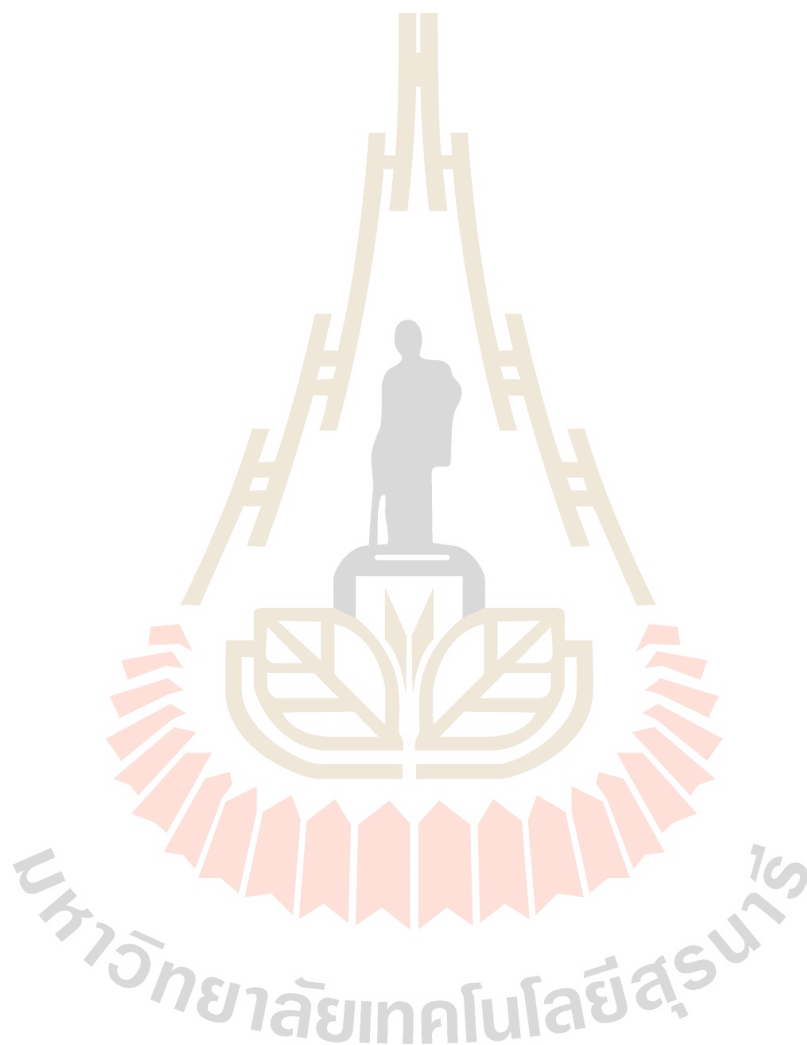
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LIST OF ABBREVIATIONS

UV-Vis	Ultraviolet-visible
NIR	Near-infrared
PDT	Photodynamic therapy
PTT	Photothermal therapy
ROS	Reactive oxygen species
PS	Photosensitizer
ISC	Intersystem crossing
ICG	Indocyanine green
MTX	Methotrexate
Aza-BODIPY	Azadipyrrromethene boron difluoride
NPs	Nanoparticles
EPR	Enhanced permeability and retention
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
DPBF	1,3-diphenylisobenzofuran
NMR	Nuclear magnetic resonance
DLS	Dynamic light scattering
FTIR	Fourier Transform Infrared Spectrometry
SEM	Scanning electron microscope
TEM	Transmission electron microscope
OCT	Optical coherence tomography
4T1	Murine breast cancer cell line
HFF	Human foreskin fibroblast cell line
HEK-293	Human embryonic kidney cell line
SOSG	Singlet oxygen sensor green
DCFH-DA	2,7-dichloro-dihydro-fluorescein diacetate
MTT	Methylthiazolyldiphenyl-tetrazolium bromide

CHAPTER I

INTRODUCTION

Cancer has become one of the greatest risks to humankind, and cancer therapy has undergone major advancements over the past few decades. Numerous researchers are attempting to discover improved strategies for reducing cancer incidence. Currently, surgery, radiation therapy, and chemotherapy are the most prevalent conventional cancer treatments (Petrelli et al., 2009; Weissleder, 2006). However, these therapies have unwanted side effects including; (i) surrounding tissue damage in radiation therapy, (ii) wound complication and poor healing from surgery, and (iii) unexpected results occurring from chemotherapy, for example, individual response to the chemo drug and more side effects such as nausea, vomiting or immune depression (Petrelli et al., 2009; Weissleder, 2006). Thus, it is critical to find an alternative or improved strategy for cure cancer.

Noninvasive treatment phototherapy includes photodynamic therapy (PDT) (Dougherty et al., 1998), which is approved by the FDA for clinical cancer therapy, and another promising modality called photothermal therapy (PTT). PDT stands out for cancer treatment and to improve the PDT's efficacy, researchers developed photosensitizers (PSs) that can generate reactive oxygen species (ROS) or singlet oxygen ($^1\text{O}_2$), which play a significant role in destroying cancer cells after they are activated by light irradiation. An advantage of utilizing light irradiation is its ability to precisely target the cancer treatment area without harming the neighboring healthy cells. Furthermore, some PSs have been utilized as imaging agents to monitor the accumulation of materials in the body, serving as a guide for cancer treatment.

However, most PSs are less soluble in water or biological media owing to their naturally high hydrophobicity from the large π -extended system and have poor tumor targeting's selectivity because of too small size. Thus, nanotechnology plays an important role in cancer research to enhance their solubility, stability, and target specificity (Bertrand, Wu, Xu, Kamaly, and Farokhzad, 2014; Lim, Lee, Zhang, and Chu, 2012; Peer et al., 2007). The development of nanomaterials has provided considerable benefits to these therapies using nanoparticles, allowing both tuning of the process and increased effectiveness. Many research studies have employed nanoparticles (NPs) loaded with PSs to improve the penetration efficacy of PSs into cancerous cells, which increased tumor accumulation through the enhanced permeability and retention (EPR) effect (Kang et al., 2020; Maeda, Bharate, and Daruwalla, 2009). The prepared NPs can efficiently enter tumors through their defective blood vessels resulting from rapid vascularization, allowing NPs to preferentially accumulate at cancer cells. Thus, the development of nanosystems to deliver imaging and/or therapeutic agents more efficiently has gained much attention in the field of nanomedicine-based therapeutic applications, both in cancer diagnosis and therapy. The goal is to design a better PS that can keep or improve its photophysical characteristics even after being further encapsulated in a nanosystem.

Therefore, we proposed the development of theranostics nanomedicines, which combine therapeutic and diagnostic applications in a single agent. In this study, we offer organic nanoparticles derived from two distinct PSs; 1) near-infrared (NIR) cyanine dye and 2) Aza-BODIPY dye (**Figure 1**), which are suitable for the development of fluorescent probes for NIR fluorescence imaging and PDT that target cancer cells via the EPR effect. For cyanine-based NPs, we use iodinated cyanine dye (**I₂-IR783**) as a PDT/NIR agent and form nanoparticles with methotrexate (MTX), a chemotherapeutic drug, to combine between PDT and chemotherapy. Another organic NP is based on Aza-BODIPY dye, we designed and synthesized a heavy-atom-free aza-BODIPY (**AB-OH**) conjugated with poly(ethylene glycol)-succinic acid polymer (mPEG-SA) that self-assembled to form stable nanoparticles (**AB-OH@mPEG-SA**). The resulting NPs were investigated for their chemical, physical, and optical properties to support their possible use in cancer imaging. Additionally, the produced NPs were evaluated in 2D

and 3D cell cultures for photocytotoxicity and cell imaging. Finally, optical coherence tomography (OCT) was employed to monitor tumor progression after treatment.

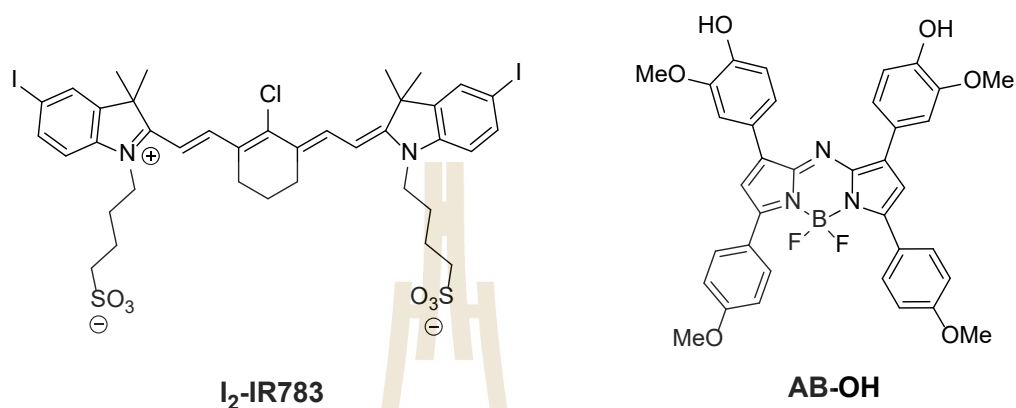


Figure 1.1 Structures of proposed photosensitizers in this thesis.

1.1 Research objectives

The ultimate goal of this thesis is to develop effective nanosystems for superior imaging-guided PDT that enhance cancer therapy *in vitro* as the following:

- 1) To develop a new photosensitizer (**AB-OH**) that has better PDT performance.
- 2) To functionalize the synthesized PSs to utilize suitable nano-delivery systems to enhance their properties.
- 3) To investigate the diagnostic effectiveness of nanomaterials, visualizing cancer cells under the confocal microscope.
- 4) To evaluate the nanomaterials' efficacy in cancer cells, which can be done by *in vitro* PDT using our synthetic materials.

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CHAPTER II

LITERATURE REVIEW

2.1 Electromagnetic radiation in medicine

Imaging techniques play a crucial role in monitoring patients' post-treatment, offering precise evaluations of whether a tumor has been fully removed or if cancer therapy is yielding the desired outcomes. This capability enables timely intervention if the disease recurs and allows for adjustments to the treatment plan based on the patient's needs. Notably, non-invasive imaging methods provide quick, often painless diagnostic assessments (Pulumati, Pulumati, Dwarakanath, Verma, and Papineni, 2023). Nowadays, a wide range of imaging techniques utilizing electromagnetic radiation from across the entire spectrum are in use, from ionizing radiation to radio waves. As displayed in **Figure 2.1**, electromagnetic radiation, especially in the form of sunlight, is a key source of many benefits for human health.

In medicine, high-frequency radiation such as gamma (γ) rays and X-rays have been widely used for applications such as cancer treatment and tumor detection. However, these forms of ionizing radiation can potentially harm DNA, making them more hazardous. In contrast, radiation from the optical range (UV, visible, and near-infrared or NIR) offers a safer alternative, with applications in both diagnostic imaging and treatments. The UV spectrum, for example, is commonly used for fluorescence-based diagnostics, skin treatments, and sterilization, but it can also cause damage depending on the wavelength (Lloyd, JJ, 2024).

Radiation in the visible and NIR range (approximately 400 nm to 900 nm) has gained increasing popularity in recent years due to its safety, affordability, and versatility. This range is especially significant in photodynamic therapy (PDT) and photothermal therapy (PTT), both of which benefit from the dual application of radiation in imaging and treatment. PDT utilizes light to activate photosensitizers, generating reactive oxygen species (ROS) that target and destroy cancer cells, while

PTT relies on NIR light to generate heat that selectively destroys tumor cells (Deng, Shao, and Zhao, 2021; Vitorino, Barros, Guedes, Caixeta, and Sabino-Silva, 2023). Moreover, researchers are focusing on developing new photosensitizers and fluorescent agents that can be activated by wavelengths beyond 1000 nm, further enhancing the potential of PDT and PTT for non-invasive cancer treatment (Luo, Zhang, Su, Cheng, and Shi, 2011; Zhu, Cheng, Chen, and Pu, 2018).

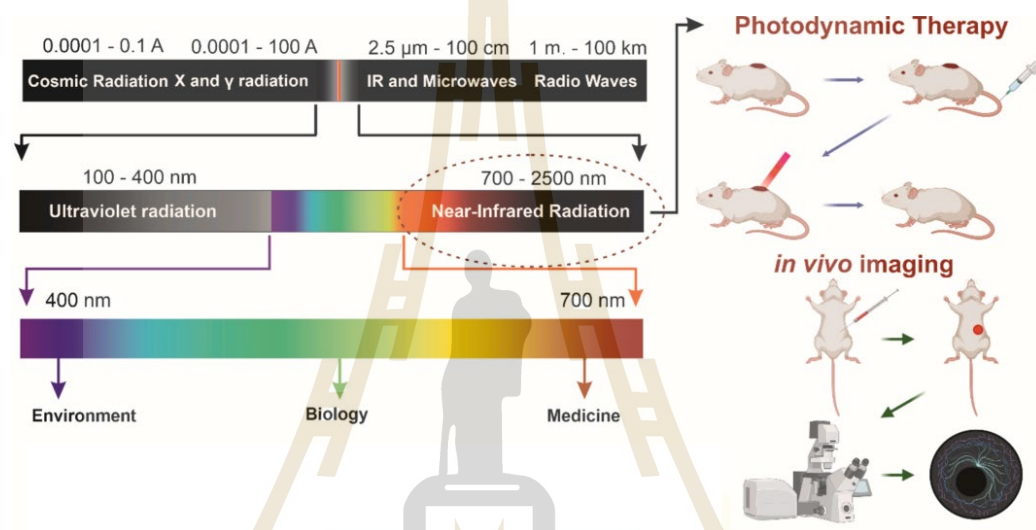


Figure 2.1 The electromagnetic spectrum is divided into various regions, with a focus on UV, visible, and NIR photons. The UV-visible range is frequently utilized in biomedicine and environmental research, whereas NIR radiation is primarily applied in photodynamic therapy and *in vivo* imaging. Copyright © 2025 Advances in Colloid and Interface Science (Paweł, Marta, and Janusz, 2025).

2.2 Photochemical mechanisms

The therapeutic properties of light have been known for thousands of years, but it was only at the beginning of the 20th century that modern photodynamic therapy (PDT) was developed (Dolmans, Fukumura, and Jain, 2003; Dougherty et al., 1998). **Figure 2.2** illustrates a simplified Jablonski diagram to reveal the potential deactivation pathways of excited states in organic photosensitizers. Generally, when light is absorbed by a light-activated agent (termed a photosensitizer, PS), it undergoes an electronic transition to a higher energy state while maintaining its spin

orientation. This excited state is unstable, and the molecule tends to return to its ground state. The excited PS can be de-excited by several pathways for relaxation. It can return to the ground state (S_0) via radiative transition in light form (fluorescence), which is utilized in fluorescence imaging, or release excitation energy as heat form (internal conversion) via non-radiative transition. Additionally, the excited molecule may transition from the singlet excited state (S_1) to the triplet state (T_1) via intersystem crossing (ISC) and then release its energy in light form (phosphorescence) (Feng, Zhang, and Ding, 2020).

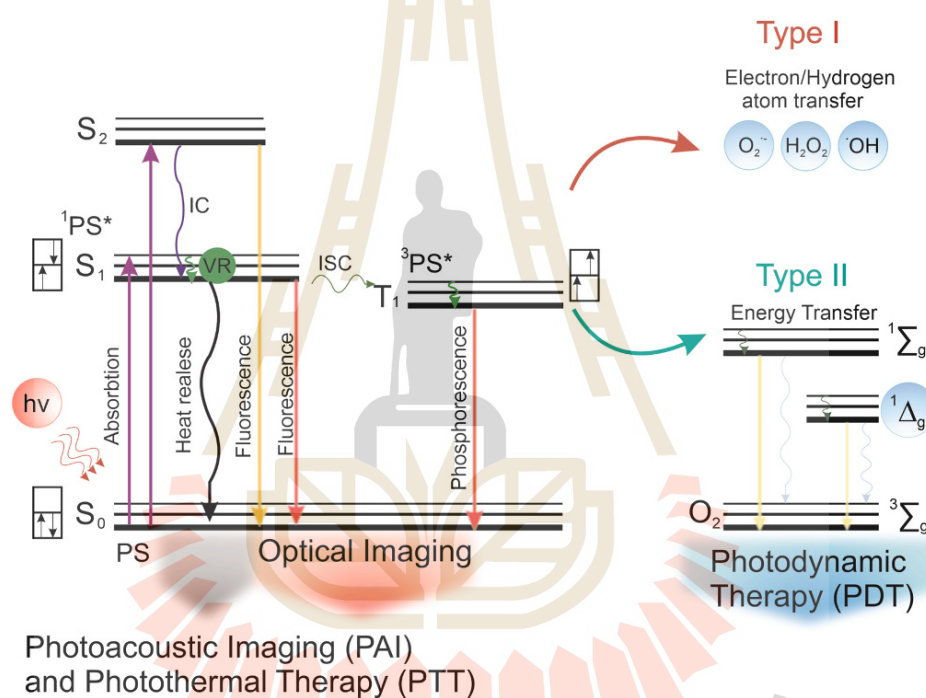


Figure 2.2 A simplified Jablonski diagram illustrates the potential deactivation pathways of excited states in organic photosensitizers. The deactivation of the singlet excited state to the singlet ground state via heat release is applied in photothermal therapy (PTT). Conversely, fluorescence emission is used in fluorescence imaging (FI). In photodynamic therapy (PDT), electron energy transfer from the triplet excited state of the photosensitizer to molecular oxygen generates reactive oxygen species (ROS). Copyright © 2025 Advances in Colloid and Interface Science (Pawet et al., 2025).

The extended lifetime of the triplet state enables photochemical reactions such as electron transfer (Type I) and energy transfer (Type II). In Type I photosensitization mechanism, the PS transfers electrons to nearby molecules, generating reactive oxygen species (ROS) like superoxide ions, hydrogen peroxide, and hydroxyl radicals, which can damage biomolecules and induce cancer cell death. Additionally, reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite (ONOO^-), can form and contribute to oxidation and nitration, further promoting cancer cell destruction and enhancing PDT efficacy. In the Type II mechanism, the PS transfers energy to molecular oxygen, producing singlet oxygen ($^1\text{O}_2$), which is critical for PDT. Nonetheless, researchers generally believe that most PSs predominantly undergo Type II reactions for cancer-targeting PDT (Agostinis et al., 2011; Carruth, 1998; Dolmans, Fukumura, and Jain, 2003).

The efficiency of these mechanisms depends on the PS structure, the oxygen concentration, and the light dose (Miao et al., 2024). In summary, the energy absorbed by a molecule, causing transitions to the singlet or triplet excited states, can be harnessed for various medical applications, including fluorescence imaging (FI), photothermal therapy (PTT), and photodynamic therapy (PDT).

2.3 Light penetration through tissues

Visible and NIR light offer an alternative method for medical imaging. The interaction between light and tissue is complex, involving absorption, scattering, diffusion, and other physical and biological processes. These interactions are influenced by the optical properties of the tissue and characteristics of the light, including absorption coefficient, photon energy, power density, and exposure time. This light-tissue interaction plays a significant role in phototherapy, medical diagnostics, and biomedical imaging (Khan, Gul, Khan, Nisar, and Ahmad, 2021; Kim and Darafsheh, 2020). The phototherapeutic window is primarily applied in PDT (**Figure 2.3A**) because it allows for deeper tissue penetration and photosensitization. NIR imaging utilizes wavelengths beyond the visible range, typically from 650 to 950 nm. This portion of the electromagnetic spectrum is known as the first optical window (NIR-I). The application of NIR light in biomedical imaging depends on

understanding photon transmission principles through tissues and optimizing the signal-to-noise ratio. As photons pass through tissue, they interact with its components, resulting in scattering, anisotropy, and light refraction. Similar effects occur during photon emission by fluorophores. Key absorptive molecules in tissues include melanin, lipids, bilirubin, vitamins, hemoglobin, and water (**Figure 2.3A**) (Hang, Boryczka, and Wu, 2022).

The depth of light penetration into tissue is a crucial factor in optical imaging, PDT, and PTT. This depth is influenced by various factors, with wavelength (λ) being particularly significant. Longer wavelengths tend to penetrate deeper into tissue, while shorter wavelengths have a more limited penetration (**Figure 2.3B**). Additionally, the optical properties of the tissue, such as absorption and scattering coefficients, have a considerable impact on light penetration.

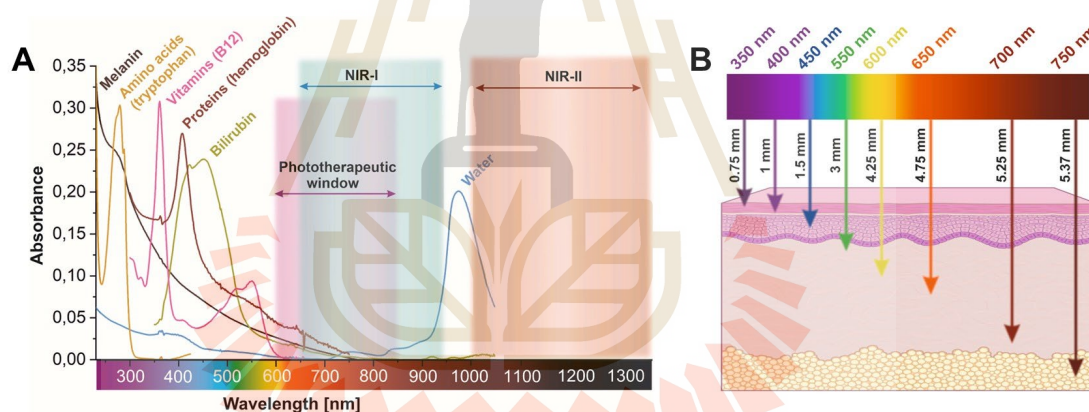


Figure 2.3 (A) Schematic representation of the phototherapeutic window for PDT (600–850 nm), displaying the electronic absorption spectra of various endogenous compounds including melanin, tryptophan, vitamin B12, hemoglobin, and bilirubin, as well as the absorption spectrum of water. (B) The light penetration through the skin. Copyright © 2024 Advances in Colloid and Interface Science (Paweł et al., 2025).

2.4 Combination of cancer imaging and treatment

Integrating cancer-targeted therapy, renowned for its enhanced therapeutic efficacy, with diagnostic capabilities for bioimaging into a single agent represents a considerable challenge; however, there has been a notable increase in interest for

developing such versatile compounds in recent years (Min and Lee, 2022). Various imaging techniques can be combined with therapeutic approaches, including the incorporation of photodynamic therapy (PDT) with non-invasive fluorescence imaging (Wang, Staudinger, Mindt, and Gasser, 2023). Many photosensitizers used in PDT are highly suitable for multimodal applications, frequently exhibiting red or near-infrared (NIR) fluorescence (Silva, Schaberle, Monteiro, Dąbrowski, and Arnaut, 2013). The primary difficulty lies in achieving an optimal balance between the optical properties, such as NIR absorption, fluorescence, or internal conversion, and the photochemical properties, such as the generation of stable triplet states, while simultaneously enhancing selectivity for tumor tissues. This balance is crucial for facilitating the compound's dual functionality in both imaging and therapy (Przygoda et al., 2023).

2.5 Photosensitizer development strategies for PDT

To develop an ideal PS for photodynamic therapy, it is necessary to consider the following criteria (Lucky, Soo, and Zhang, 2015):

- 1) The PS itself should be nontoxic, even at relatively high levels in the absence of light, but exhibiting toxicity only upon light irradiation, since the PS could be internalized by some excretory organs, such as the liver and kidney.
- 2) The PS should have outstanding triplet state generation efficiency, which is essential to produce an enormous amount of ROS or $^1\text{O}_2$.
- 3) The PS must be excited with intense NIR absorption (700-1000 nm), which is beneficial for deep-seated tumor treatment.
- 4) Amphiphilicity should also be considered, as it requires both hydrophilicity for intravenous injection and lipophilicity for tumor cell binding.
- 5) An ideal PS should have tumor-targeting ability, which is important for effective tumor accumulation, improved therapeutic efficacy, and minimized side effects.

Recently, the development of PSs mainly focuses on the red shift of the absorption wavelength, better tumor-targeting ability, and higher photostability. It is achieved by decorating PSs with bioactive agents (e.g., peptides, antibodies, and

antisense) or chemical conjugation /loading/encapsulation of existing PSs in nanosystems.

2.5.1 Cyanine dyes

In the past decade, near-infrared (NIR) fluorescent cyanine dyes have been intensively investigated as PDT agents (Shi, Wu, & Pan, 2016), the Food and Drug Administration (FDA) approved NIR dye for medical applications in the early 1950's. These PSs are suitable for millimeter-level tumor detection because the light in this region has low background fluorescence from endogenous molecules, low autofluorescence from biological materials, and low light scattering (Luo et al., 2011; Weissleder, 2001). Furthermore, these dyes could be used as drug carriers to safely deliver chemotherapy medications to malignancies (Cooper et al., 2021). However, direct usage of NIR cyanine, such as indocyanine green or ICG ($\lambda_{\text{ex}} = 808 \text{ nm}$, $\lambda_{\text{em}} = 830 \text{ nm}$) (Alander et al., 2012), has certain restrictions due to poor fluorescence quantum yield and a short half-life of around 2–4 min (Mindt, Karampinis, John, Neumaier, & Nowak, 2018). In addition, ICG has an extremely low singlet oxygen quantum yield ($\Phi = 0.077$) which limits its practical utility. Singlet oxygen generation in PDT involves energy transfer from the sensitizers excited triplet state to the ground state of molecular oxygen. Excited triplet states are formed from excited singlet states via a process known as intersystem crossing (ISC). As fluorescence occurs because of radiative deactivation from a fluorophores excited singlet state, the fluorescence and singlet oxygen quantum yields are invariably linked, with good fluorophores typically being poor sensitizers.

2.5.2 Modification of Cyanine dye for PDT applications

Iodinated cyanine dye (**I₂-IR783**) was recently developed (Atchison et al., 2017) with high photostability and strong fluorescence, which made them more suitable than conventional cyanine dyes as fluorescence probes. The addition of heavy atoms, such as iodine or bromine, to the structural skeleton of conjugated aromatic,

Azadipyrrromethene boron difluoride (aza-BODIPY) derivatives are recently being developed as one of the most promising types of PSs for PDT due to their unique features, including high fluorescence quantum yields (suppressed nonradiative decay), tunable absorption/emission from visible light to near-infrared, high photostability and chemical stability (Chen et al., 2018; Tian et al., 2022; Treekoon et al., 2021; Wu et al., 2019; Yu et al., 2020; M. Zhao et al., 2018). Meanwhile, they also present better photostability over some organic photosensitizers, such as porphyrins, cyanines, and squaraines, which allows better biosafety and therapeutic efficacy during phototherapy (Cai et al., 2018).



Azadipyrromethene boron

Typically, the development of aza-BODIPY PSs with photodynamic characteristics includes heavy atoms to efficiently facilitate the ISC and consequently generate ROS, but they still suffer some drawbacks, such as increased dark toxicity, short triplet state lifetimes, and low photostability, which hinder their clinical and

Typically, the development of aza-BODIPY PSs with photodynamic characteristics includes heavy atoms to efficiently facilitate the ISC and consequently generate ROS, but they still suffer some drawbacks, such as increased dark toxicity, short triplet state lifetimes, and low photostability, which hinder their clinical and

biomedical applications. Therefore, considering novel aza-BODIPY PSs without the use of heavy atoms is of great importance for their potential applications in the clinic (J. Zhao, Xu, Yang, Wang, and Zhong, 2015).

2.5.4 Heavy-atom-free Aza-BODIPY

Heavy-atom-free PSs exhibited many advantages over traditional PSs, including (1) high ROS generation capabilities due to their excellent triplet harvesting, (2) low dark toxicity, (3) long-lived triplet excited states, (4) good photostability and biocompatibility, (5) easy modification, and (6) low cost (Nguyen, Yan, Zhao, and Yoon, 2020). Reducing the singlet–triplet energy gap (ΔE_{ST}) is one of the strategies for developing heavy-atom-free PSs (Figure 2.5). Therefore, a PS with a small singlet–triplet energy gap could be considered a promising candidate for PDT.

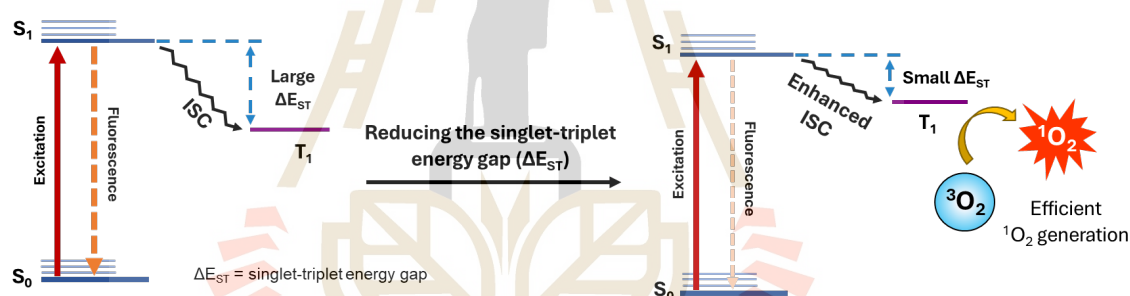


Figure 2.5 Proposed strategy of triplet state formation and 1O_2 generation by reducing the singlet–triplet energy gap (ΔE_{ST}).

For instance, Liu et al. introduce a novel supramolecular approach to construct nano-photosensitizers (nano-PSs) from the metastable aggregation of heavy-atom-free Aza-BODIPY dyes (**BDP-1** and **BDP-2**) featuring pyridine units (Yi-chen, Yuan, Guang-jian, and Guo-wen, 2023). These nano-PSs demonstrate high singlet oxygen (1O_2) generation efficiency (up to 0.85–0.89), addressing several inherent limitations of traditional heavy-atom Aza-BODIPY photosensitizers. X-ray crystallographic analysis and theoretical calculations revealed that intermolecular electrostatic interactions induced the formation of J- and H-aggregates, which altered

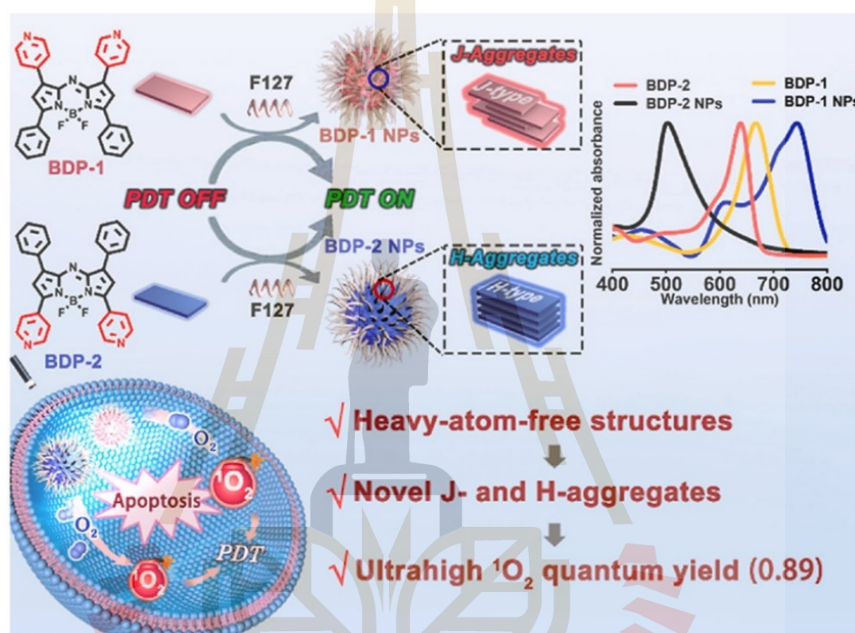


Figure 2.6 A supramolecular approach is proposed for creating metastable J- and H-aggregates of Aza-BODIPY dyes through weak intermolecular hydrogen bonding between adjacent molecules. These aggregates can be directly formed during nanoparticle fabrication, providing stable phototherapeutic efficacy. Copyright © 2023 Dyes and Pigments (Yi-chen et al., 2023).

In another study (Miao et al., 2024), a donor-excited photoinduced electron transfer (d-PeT)-driven singlet oxygen charge transfer-intersystem crossing (SOCT-ISC) mechanism is employed to develop heavy-atom-free PSs for PDT of tumors. This is achieved by directly incorporating the electron-deficient N-alkylquinolinium unit (as an electron acceptor) into the meso-position of a near-infrared (NIR) distyryl Bodipy

chromophore (as an electron donor). In less polar environments, the PSs exist as monomers, facilitating the production of singlet oxygen ($^1\text{O}_2$) through Type-II PDT, driven by the d-PeT-mediated population of the triplet excited state via SOCT-ISC. In contrast, in aqueous environments, the PSs form nanoaggregates, leading to the generation of superoxides ($\text{O}_2^{\cdot-}$) and hydroxyl radicals ($\text{HO}^{\cdot}$) via d-PeT-driven formation of a delocalized charge-separated state (Type-I PDT). This developed PS demonstrates effective cancer cell killing and tumor ablation without harming normal cells or tissues under NIR light irradiation, as confirmed *in vitro* and *in vivo*. This study broadens the design scope of PSs by incorporating the d-PeT mechanism, offering significant potential for the development of novel PSs for tumor PDT (Figure 2.7).

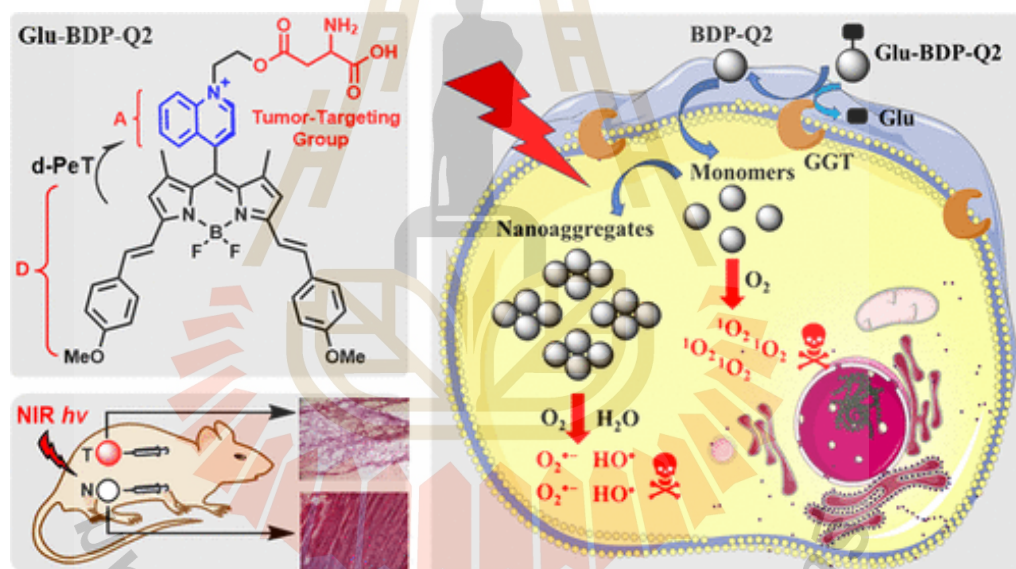


Figure 2.7 Constructing Aza-BODIPY-based heavy-atom-free PS for PDT using the spin-orbit charge transfer intersystem crossing (SOCT-ISC), is driven by the acceptor-excited photoinduced electron transfer (a-PeT). Copyright © 2024 ACS Applied Materials & Interfaces (Miao et al., 2024).

2.6 Nanoparticles in drug delivery

Most of the developed PSs usually lack hydrophilicity and tumor-targeting ability. Hence, many researchers encapsulated the PSs in a polymer to create nanoparticles (NPs), which have been shown to improve blood circulation and thus tumor accumulation (Tian et al., 2022; Treekoon et al., 2021). There are two primary methods for delivering drugs into solid tumors using nanomaterials: (i) active targeting, which involves conjugating targeting ligands that bind to overexpressed receptors on tumor cells, and (ii) passive targeting, which leverages the enhanced permeability and retention (EPR) effect (Hiroshi Maeda, Bharate, and Daruwalla, 2009). However, this literature review focuses specifically on passive targeting for cancer diagnosis.

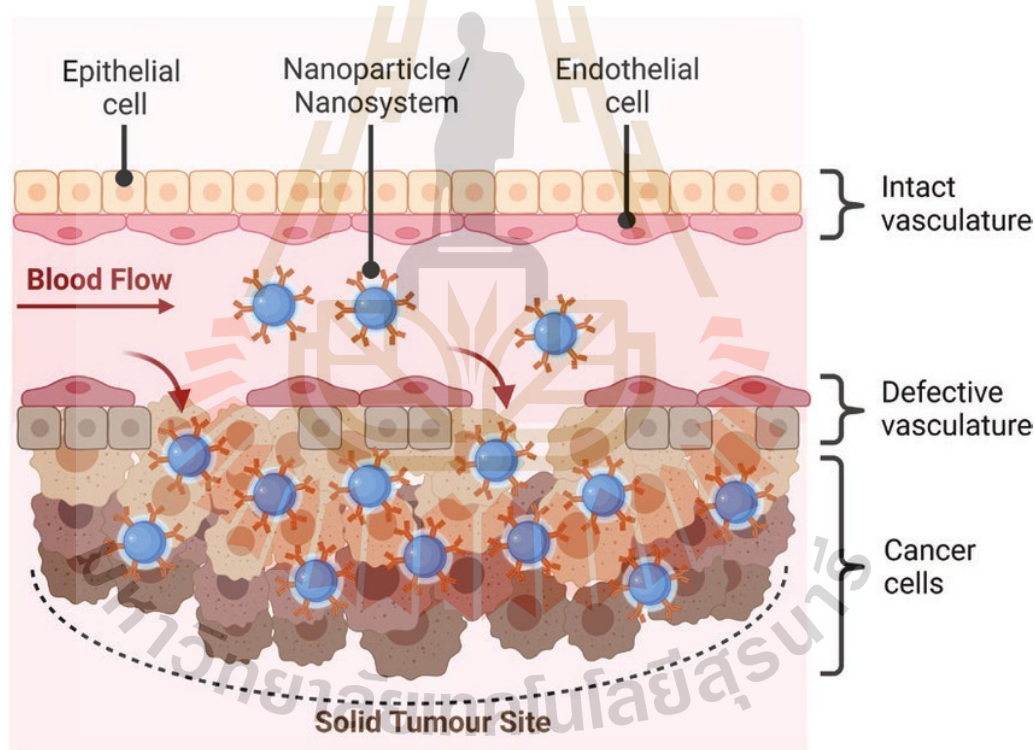


Figure 2.8 Schematic illustration of the enhanced permeability and retention (EPR) effect in a tumor site. Copyright © 2024 RPS Pharmacy and Pharmacology Reports (Baskaran et al., 2025).

EPR offers a strong rationale for the development of nano-sized drug carriers, as it allows particles to selectively accumulate in tumors due to their size and extended circulation time. A notable characteristic of tumors is their rapid and uncontrolled growth, which creates an increased demand for oxygen and nutrients. When solid tumors reach a size of 1–2 mm in diameter, the surrounding normal blood vessels are no longer sufficient to support their continued expansion. As a result, intratumoral angiogenesis becomes essential for their further growth (Iyer, Khaled, Fang, and Maeda, 2006; Hiroshi Maeda, Fang, Inutsuka, and Kitamoto, 2003). Due to the rapid creation of new blood vessels, blood vessel breaches allow NPs to infiltrate the tumor tissue (Hiroshi Maeda et al., 2009), as indicated in **Figure 2.8**. The formulation of those PS into nanoscale materials can lead to a high accumulation of PS in the tumor sites via EPR effects thus enhancing PDT efficiency (Cai et al., 2017; Taratula, Schumann, Duong, Taylor, and Taratula, 2015).

In addition to the physiological characteristics of tumors and their microenvironments, the physicochemical properties of macromolecules or nanoparticles—such as size, shape, surface features, and biocompatibility—significantly influence the efficiency of EPR-based tumor accumulation (Jain and Stylianopoulos, 2010; H. Maeda, 2021). For instance, nanoparticles with hydrodynamic diameters of 5–6 nm are typically cleared quickly from circulation, while nanoparticles larger than 200 nm face challenges in extravasating into tumors. Thus, nanoparticles with diameters ranging from 50 to 150 nm are considered optimal for EPR-mediated tumor targeting (Davis, Chen, and Shin, 2008). Recent studies also suggest that nanoparticles with high aspect ratios and low surface curvature, such as rod-shaped, discoidal, or worm-like particles, are better able to evade phagocytosis, leading to longer circulation times and greater tumor accumulation compared to spherical nanoparticles (Blanco, Shen, and Ferrari, 2015; Kinnear, Moore, Rodriguez-Lorenzo, Rothen-Rutishauser, and Petri-Fink, 2017). Additionally, surface charge plays a crucial role in the intratumoral distribution of nanoparticles via the EPR effect. Nanoparticles with high positive charges tend to be captured by the vascular endothelium due to interactions with negatively charged phospholipids, while those with high negative charges are more likely to be cleared by the liver, spleen, or other parts of the

reticuloendothelial system (RES). Therefore, the optimal surface charge for nanoparticles is typically neutral or slightly negative (Fang, Islam, and Maeda, 2020).

2.6.1 Cyanine dye-based nanoparticles for phototherapy

Cyanine dyes often face solubility issues and tend to form aggregates under physiological conditions. Furthermore, their short residence time at tumor sites and limited photostability hinder their effectiveness. Nanoparticles (NPs), with their large surface area, excellent biocompatibility, and ease of surface modification, have been widely employed in PDT and PTT to overcome these limitations of photosensitizers. Cyanine nanoparticles (Cyanine-NPs) have diverse applications in imaging techniques (such as near-infrared fluorescence, photoacoustic, and magnetic resonance imaging) to guide combination therapies, including PDT/PTT, and PTT/chemotherapy. Many systems based on cyanine nanoparticles for combination therapy have been developed, and research in this area is rapidly advancing (Qian, Si-Rui, and You-Quan, 2024).

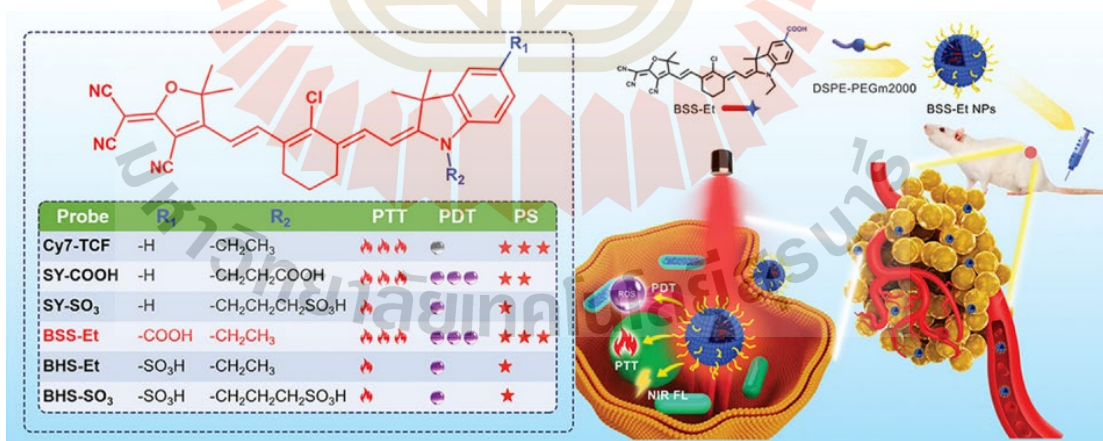


Figure 2.9 Schematic diagram of synthesis route and anti-tumor mechanism of BSS-Et NPs for synergistic cancer phototherapy. Copyright © 2023 Advances Healthcare Materials (Liu et al., 2023).

Wang et al. developed an asymmetrical heptamethine cyanine by adding a sulfonic acid or carboxyl acid group to the indole ring or branch chain, creating a new series of photosensitizers (**Figure 2.9**). The BSS-ET nanoparticles (NPs) were formed through self-assembly with DSPE-PEGm2000. Photophysical studies demonstrated that the BSS-ET NPs had high ROS production and heat generation capabilities. These NPs facilitated NIR fluorescence image-guided PDT/PTT combination therapy by passively targeting 4T1 mouse tumors through the EPR effect (Liu et al., 2023).

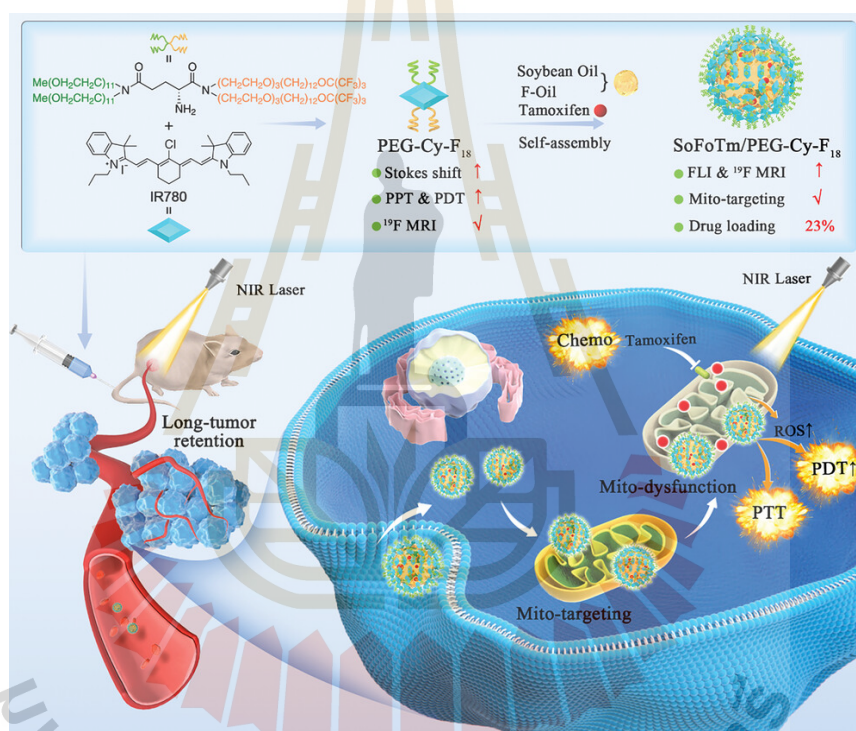


Figure 2.10 Schematic illustration of versatile theranostic SoFoTm/PEG-Cy-F18 nanoparticles. Copyright © 2023 Advances Healthcare Materials (Li et al., 2023).

Chen et al. utilized the cyanine dye IR780 as the photosensitizer and combined it with PEG-Cy-F18, a chemotherapy drug tamoxifen (TAM), and 1,11-diperfluoro-tert-butoxyundecane (F-oil) to form stable nanoparticles (**SoFoTm/PEG-Cy-F18**). These nanoparticles were then employed for NIR dual-modal imaging-guided

chemotherapeutic, photothermal, and photodynamic therapy (chem-PTT-PDT) targeting breast cancer (Figure 2.10) (Li et al., 2023).

2.6.2 Aza-BODIPY-based nanoparticles for phototherapy

For nanoparticles based on Aza-BODIPY dyes, encapsulating PSs within functional amphiphilic polymers such as polyethylene glycol (PEG) resulted in the development of polymeric-based aza-BODIPY NPs for cancer theranostic applications have been widely developed (Chen et al., 2018; Tian et al., 2022; Treekoon et al., 2021).

Treekoon et al. developed iodo-substituted aza-BODIPY (AZB-I) encapsulated nanoparticles, incorporating the amphiphilic poly(ethylene glycol)-block-poly(ϵ -caprolactone) (PEG-*b*-PCL) polymer for a targeted drug delivery system (Treekoon et al., 2021). The resulting AZB-I@PEG-*b*-PCL nanoparticles exhibited a uniform spherical shape with hydrodynamic average sizes between 44.6 and 48.2 nm. Additionally, these PSs proved effective in treating 4T1 breast cancer cells when exposed to 660 nm red LED light (Figure 2.11).

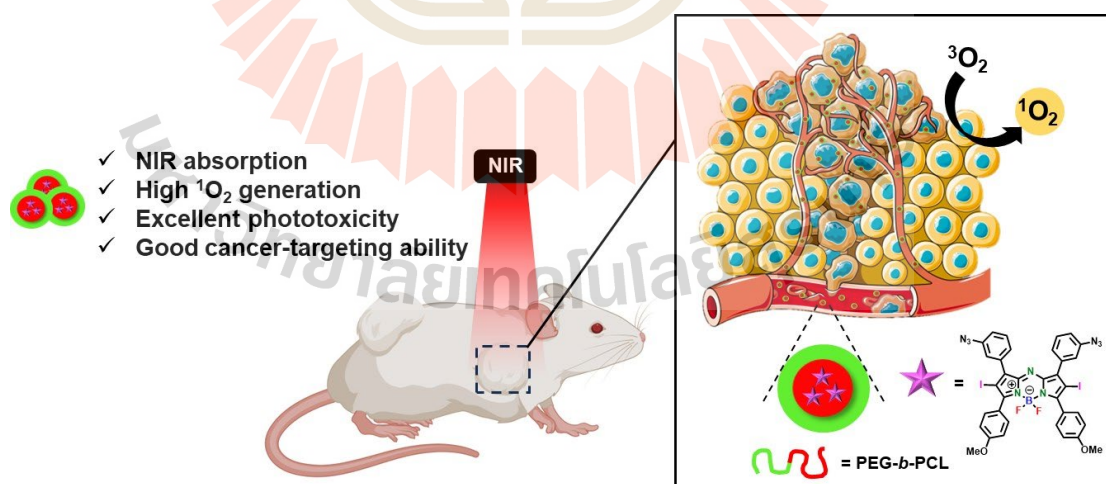


Figure 2.11 Schematic illustration of AZB-I@PEG-*b*-PCL nanoparticles in mice model. Copyright © 2021 Materials Chemistry Frontiers (Treekoon et al., 2021).

Dong et al. developed a near-infrared (NIR) aza-BODIPY derivative, **MeOABBr** ($\Phi_{\Delta} = 84\%$), and encapsulated it with polyethylene glycol–folic acid (PEG–FA) and polyethylene glycol–triphenylphosphonium (PEG–TPP) to form dual-targeting nanoparticles for both tumors and mitochondria, designated as **FMAB NPs** (Chen et al., 2018). Upon exposure to NIR light, these **FMAB NPs** effectively produce reactive oxygen species (ROS) and generate heat, disrupting mitochondrial function and leading to cell apoptosis. Furthermore, **FMAB NPs** possess distinctive optical properties, enabling precise monitoring via photoacoustic, fluorescence, and photothermal imaging *in vivo*. Notably, both *in vitro* and *in vivo* studies have demonstrated that these dual-targeted **FMAB NPs** exhibit high phototherapeutic efficacy while showing minimal toxicity to normal tissues (Figure 2.12).

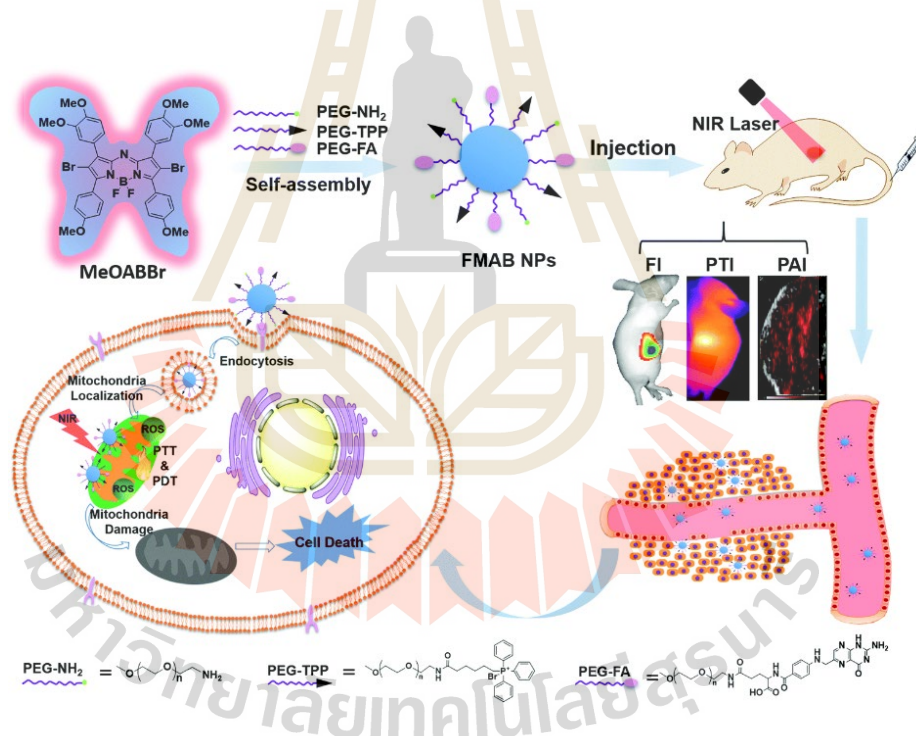


Figure 2.12 Schematic illustration of **FMAB NPs** for multimodal imaging-guided phototherapy. Copyright © 2018 Journal of Materials Chemistry B (Chen et al., 2018).

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CHAPTER III

HYBRID CYANINE/METHOTREXATE NANOPARTICLES FOR SYNERGISTIC PDT/CHEMOTHERAPY OF BREAST CANCER

3.1 Abstract

Nanomedicine is generally developed by using nanocarriers to transport specific therapeutic agents. In this study, a carrier-free nanosystem for imaging and combining photodynamic therapy (PDT) with chemotherapy was designed through the simple self-assembly of a cyanine dye (**I₂-IR783**) and a chemotherapy drug (methotrexate, MTX). The resulting nanoparticles (**I₂-IR783/MTX@NPs**) demonstrated a spherical shape with a size of 240.6 ± 2.5 nm. These nanoparticles were effectively internalized by 4T1 murine breast cancer cells and exhibited a synergistic anticancer effect when exposed to near-infrared (NIR) light. Furthermore, the 3D tumor model displayed similar phototoxicity to the nanoparticles as observed in 2D cell cultures. The PDT effectiveness of the nanosystem in a physiological environment was validated by detecting intracellular reactive oxygen species and conducting a live/dead viability/cytotoxicity assay after NIR light exposure. Additionally, optical coherence tomography (OCT) was employed as an alternative method to assess the treatment response. These findings highlight the potential of **I₂-IR783/MTX@NPs** for theranostic applications in breast cancer PDT-chemotherapy.

3.2 Introduction

Heptamethine cyanine (Hcyanine) dyes are extensively utilized for both cancer detection and treatment (Hu et al., 2020; Shi, Wu, and Pan, 2016). These dyes, which are fluorescent in the near-infrared (NIR) range, are particularly effective for detecting tumors at millimeter depths. This is because NIR light experiences minimal interference from fluorescence background of endogenous molecules, reduced auto-

fluorescence from biological tissues, and less scattering of light (Alander et al., 2012; Weissleder, 2001). By incorporating heavy halogen elements, such as iodine or bromine, into the molecular framework of these dyes, they can act as powerful agents for photodynamic therapy (PDT) (Shi et al., 2016). These halogen atoms enhance the intersystem crossing (ISC) efficiency, enabling the dye to reach its triplet state and produce singlet oxygen, which is key for effective cancer treatment (Bodio, Denat, and Goze, 2019; He et al., 2016). Additionally, these dyes can function as carriers for chemotherapy drugs, delivering them safely to cancerous tissues (Cooper et al., 2021). However, the clinical use of Cyanine dyes, like indocyanine green (ICG), is limited by challenges such as poor fluorescence quantum yield and a short half-life of only 2-4 minutes (Mindt, Karampinis, John, Neumaier, and Nowak, 2018).

Nanoparticle-based theranostic probes, which integrate optical diagnostics with phototherapy, have gained significant attention in drug delivery systems due to their ability to enhance drug stability, extend circulation time, control drug release, improve biodistribution, reduce side effects, and increase tumor accumulation through the EPR effect (Bertrand, Wu, Xu, Kamaly, and Farokhzad, 2014; Cheng et al., 2015; Elsadek and Kratz, 2012; Liang, Wei, Evans, and Duan, 2014; Peer et al., 2007; Qian et al., 2016; Yan et al., 2013; Yang et al., 2016; Yao et al., 2017). Self-assembly techniques have been widely employed in drug delivery to boost efficacy while minimizing side effects (Chen, Xu, Chen, and Zhang, 2014; Cheng et al., 2015; Choi, Swierczewska, Lee, and Chen, 2012; Reimer, Pos, Thines, Grün, and Bode, 2011; Shen et al., 2011). However, complex synthetic procedures have limited the clinical application of these nanomedicines, and the use of carriers like polymers or lipids to encapsulate drugs within nanostructures often leads to suboptimal drug loading efficiency (Y. Shamay et al., 2018; Sun, Zhou, Qiu, and Shen, 2017).

Recently, carrier-free nanoformulations have been explored to study various nanomaterial-based cyanine dyes. Heller et al. reported that a group of sulfated indocyanine dyes (IR783) could self-assemble with hydrophobic drugs to form stable, tumor-targeted nanoparticles (Y. Shamay et al., 2018). They found that these indocyanine nanoparticles could achieve a high drug encapsulation efficiency, with up to 90% by mass. In another study, Shen et al. developed a method to directly

self-assemble indocyanine green (ICG) with the clinically approved hydrophobic drug SN38 for combined chemotherapy and phototherapy, with the resulting nanoparticles showing excellent imaging capabilities and enhanced therapeutic effects (Hu et al., 2020). Thus, the development of carrier-free nanomedicines for use as theranostic probes is a promising area of focus for clinical applications.

Methotrexate (MTX, 4-amino-10-methylfolic acid) is one of the early generations of anticancer drugs, serving as a folic acid analogue in the treatment of breast cancer (Koźmiński, Halik, Chesori, and Gniazdowska, 2020). MTX works by inhibiting dihydrofolate reductase (DHFR), leading to the accumulation of polyglutamated folates that further inhibit thymidylate synthase and glycinamide ribonucleotide formyltransferase. This results in a shortage of reduced folate substrates, which in turn inhibits purine nucleotide synthesis, reducing DNA synthesis and slowing cell proliferation (Genestier, Paillot, Quemeneur, Izeradjene, and Revillard, 2000). However, due to its low water solubility, MTX has limited bioavailability and efficacy. Its low molecular weight also results in a lack of selectivity and a short half-life in the bloodstream (Attari, Nosrati, Danafar, and Kheiri Manjili, 2019). Previous studies have shown that simply delivering chemotherapeutic drugs to target cells is insufficient to induce cell death, and high-dose MTX can cause significant side effects in patients (Adam de Beaumais and Jacqz-Aigrain, 2012). Additionally, tumor cells often develop resistance to chemotherapy, including MTX resistance, which has been extensively studied in various research areas (Boratyński, Opolski, Wietrzyk, Górski, and Radzikowski, 2000; Lindgren et al., 2006; Pignatello et al., 2004; Purcell and Ettinger, 2003; Ryser and Shen, 1978; Stehle et al., 1997).

Compared to ICG, iodinated cyanine dye (**I₂-IR783**) has a higher singlet-oxygen quantum yield, making it a more effective choice for photodynamic therapy. To address MTX resistance in tumor cells, we developed a carrier-free nanotheranostic agent in this study for synergistic PDT/chemotherapy and NIR fluorescence imaging. **I₂-IR783** was used as the PDT/NIR agent to form nanoparticles with the chemotherapeutic drug, MTX. The resulting nanoparticles (**I₂-IR783/MTX@NPs**) were analyzed for their chemical, physical, and optical properties to assess their potential for cancer imaging. Through synergistic therapy for breast cancer, we demonstrated

the strong anticancer activity of **I₂-IR783/MTX@NPs**, showing that the combination therapy could overcome MTX resistance in breast cancer at high doses. Additionally, the nanoparticles were tested in a 3D tumor cell model, evaluating photocytotoxicity, cell imaging, and optical coherence tomography (OCT) to track tumor progression after therapy.

3.3 Experimental

3.3.1 Materials and instruments

All reagents were purchased from various manufacturers (Sigma Aldrich, TCI, Carlo Erba, and Merck) and used without further purification. All column chromatography was performed with the use of Merck Silica gel (230-400 mesh). The nuclear magnetic resonance (NMR) spectra were recorded on a 500 MHz (¹H) and 125 MHz (¹³C) NMR spectrometer (Avance III, Spectrometer, Bruker Biospin Ag, USA). A Bruker Microflex MALDI-TOF mass spectrometer was used to obtain the high-resolution mass spectra of the samples. A Cary Series UV-Vis-NIR spectrophotometer was used to collect UV-vis-NIR absorption spectrum data (Agilent Tech, Santa Clara, CA, USA). Emission spectra were recorded using a PTI QuantaMaster 500 Near Infrared Photoluminescence System (HORIBA Scientific, USA). Dynamic light scattering (DLS) measurement was measured by the Zetasizer Nano ZS (Malvern Panalytical). Transmission electron microscope (TEM) images were taken on a JEM-2100-Plus Transmission Electron Microscope. DLS and TEM were used to determine the size of NPs. Fourier transform infrared spectrophotometer (FTIR, PerkinElmer Spectrum GX) was utilized to collect IR spectra. To confirm the 3D structure of the cultured cells, 3D microscopic imaging of the tumor cells model inside the microplate was performed by using a custom-built frequency domain optical coherence tomography (FD-OCT).

3.3.2 Synthesis of **I₂-IR783** and its nanoparticles preparation

Firstly, the iodinated cyanine dye (**I₂-IR783**) was synthesized according to the literature report (Usama, Thavornpradit, and Burgess, 2018), as illustrated in **Figure 3.1**.

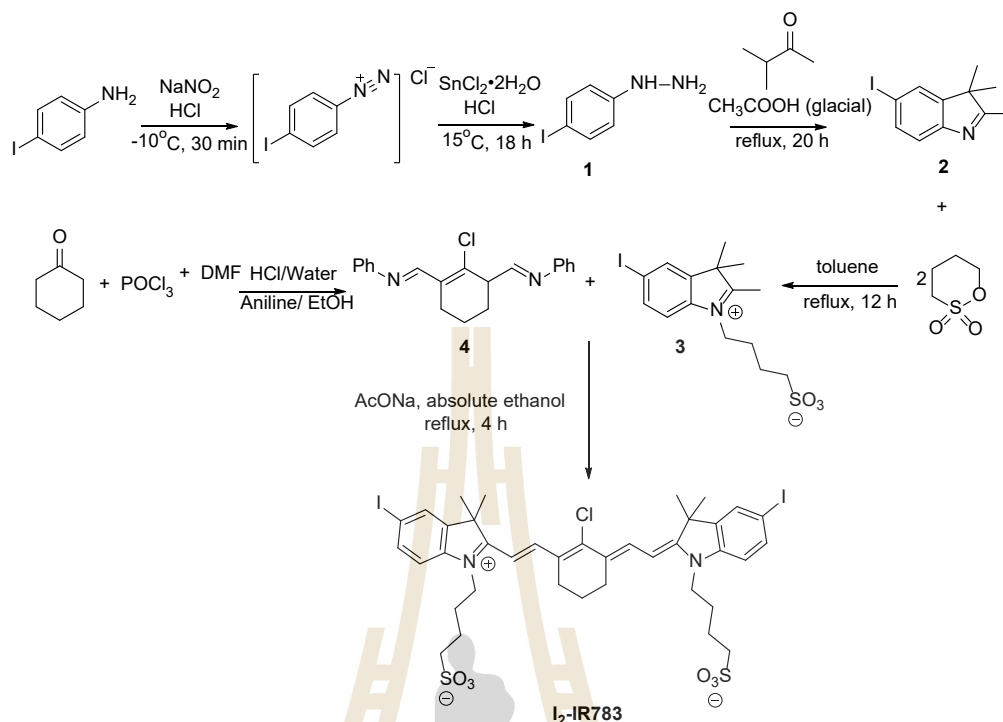


Figure 3.1 Synthetic route of I₂-IR783.

After that, cyanine/methotrexate nanoparticles (I₂-IR783/MTX@NPs) were prepared using a modified nanoprecipitation method (Hu et al., 2020; Y. Shamay et al., 2018). Methotrexate (100 μ L) was dissolved in dimethyl sulfoxide (DMSO, 1 mg/mL) and then added dropwise (20 μ L every 15 seconds) into an aqueous solution of I₂-IR783 (600 μ L, 0.5 mg/mL in 50 μ M sodium bicarbonate) with gentle vortexing. The resulting solution was then centrifuged at 13,000 rpm and 4 $^{\circ}$ C for 60 minutes. The pellet was washed three times with PBS and resuspended in 100 μ L of sterile phosphate-buffered saline (PBS, pH = 7.4) to obtain the I₂-IR783/MTX@NPs. The prepared nanoparticles were stored at 4 $^{\circ}$ C for future use. The amounts of the drug and the free dye in the nanostructure were calculated from calibration curves of free dye or drug using a UV-Vis-NIR spectrophotometer (Shimadzu/UV-1900i).

3.3.3 Photophysical properties of I₂-IR783/MTX@NPs

A stock solution of I₂-IR783/MTX@NPs containing 4 μ M of I₂-IR783 was prepared in PBS. Then, 200 μ L of the stock solution was added to 3.0 mL of PBS containing 3% Tween 80 in a 3.5 mL quartz cuvette, resulting in a final concentration

of 0.267 μM . The UV–Vis–NIR absorption was measured in a Cary Series UV–vis–NIR spectrophotometer. The fluorescence spectra were recorded on a PTI QuantaMaster 500 Near-Infrared Photoluminescence System (HORIBA Scientific) with an excitation wavelength of 785 nm, an excitation slit width of 10 nm, and an emission slit width of 10 nm.

3.3.4 Characterization of nanoparticles

For the characterization of prepared NPs, the particle size was determined by dynamic light scattering (DLS) (Malvern Model Zetasizer-ZS). The morphology of obtained NPs was investigated by JEM-2100-Plus transmission electron microscope (TEM; JEOL). The concentration of the NPs was calculated from UV–vis–NIR absorbance based on a calibration curve of free dye (Shimadzu/UV-1900i).

3.3.5 Singlet oxygen quantum yield determination

Indirect measurement of singlet oxygen generation was evaluated by reaction with 1,3-diphenylisobenzofuran (DPBF), singlet oxygen scavenger, as a probe. The solutions of ICG, free $\text{I}_2\text{-IR783}$, and $\text{I}_2\text{-IR783/MTX@NPs}$ were prepared in PBS (3% Tween-80) at a concentration of 2 μM . DPBF (1 mM) was prepared in DMSO and diluted to 30 μM in buffer solutions. Then, the solutions were irradiated by an 805 nm LED lamp (at a power density of 8.7 mW cm^{-2}) every 1 min for 10 min. The decreasing absorbance at 415 nm was measured in a UV-vis spectrophotometer (Shimadzu/UV-1900i). The rate of change of absorbance was plotted against irradiation time. This method was also employed for free $\text{I}_2\text{-IR783}$ at the same concentration. The singlet oxygen quantum yield was calculated according to Eq. 2,

$$\Phi_x = \Phi_{\text{st}}(\text{grad}_x / \text{grad}_{\text{st}})(F_{\text{st}} / F_x)$$

Φ_{st} represents the quantum yield of the standard; Φ_x represents the quantum yield of the unknown, and grad is the slope of the best linear fit line. F stands for the absorption correction factor ($F = 1 - 10^{-\text{abs}}$; abs represents absorbance), and subscripts x and st denote the unknown and the standard, respectively.

3.3.6 Cell culture

Murine breast cancer (4T1) cells and human foreskin fibroblast (HFF) cells were cultured in 75 cm² culture flasks using media (Roswell Park Memorial Institute (RPMI-1640, HYCLONE) for 4T1 cells and Dulbecco's Modified Eagle's Media (DMEM, HYCLONE) for HFF cells), supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% Penicillin-Streptomycin Solution 100x (CORNING). All the cells were cultured at 37 °C in a humidified 95% air, 5% CO₂ atmosphere.

3.3.7 Live-cell imaging

4T1 and HFF cells were seeded in an 8-well Chambered Coverglass (Nunc Lab-Tek II Chamber Slide) with non-removable wells at a density of 7×10^3 cells per well and incubated for 24 hours at 37 °C under 5% CO₂. After incubation, the cell culture media was removed, and solutions of free dye and nanoparticles (NPs) in complete medium were added for various incubation times (0, 1, 3, 6, 12, and 24 hours). The cells were then washed three times with PBS and stained with 1.0 μ M Hoechst 33342 (Thermo Fisher Scientific) for 10 minutes. All cells were visualized in a Laser Scanning Confocal Microscope (LSCM, Nikon A1Rsi) with a 60X oil immersion objective lens. The photosensitizers (PSs) were excited with a 641 nm laser (Em: 663-738 nm), while Hoechst 33342 was excited with a 405 nm laser (Em: 425-475 nm). All data were analyzed using ImageJ, and the quantitative fluorescent intensity was presented as corrected total cell fluorescence (CTCF), with values expressed as mean $\pm$ SD (n = 30, 30 cells/set).

3.3.8 Light-induced cytotoxicity

Cell viability was tested using the MTT assay (Van Meerloo, Kaspers, and Cloos, 2011). First, 4T1 and HFF cells were seeded into 96-well culture plates at a density of 1×10^4 cells per well and incubated for 24 h. The cells were then treated with free MTX, free I₂-IR783, and I₂-IR783/MTX@NPs containing various concentrations of I₂-IR783 (0, 1.25, 2.5, 5, 10, and 20 μ M) in complete culture medium for 24 h. After incubation, the cells were washed with 0.01 M PBS buffer (pH 7.4), and fresh complete cell culture medium was added before the cells were irradiated with an 805 nm LED lamp for 30 minutes, followed by re-incubation for an additional 24 h. Subsequently, the cells were treated with methylthiazolyldiphenyl-

tetrazolium bromide (MTT reagent, 20 μL , 0.5 mg/mL, Sigma-Aldrich) for 2.5 h. After media removal, dimethyl sulfoxide (DMSO) was added to dissolve the formazan product. The cell's viabilities were detected through UV-vis absorption of formazan at 560 nm wavelength in a microplate reader (BMG Labtech/SPECTROstar Nano), and % viability was calculated as $100 \times (A_{\text{sample}}/A_{\text{control}})$; where A = absorbance at 560 nm.

3.3.9 Intracellular ROS and singlet oxygen generation

To visualize intracellular singlet oxygen generation, 4T1 cells were seeded on an 8-well chambered coverglass (1×10^4 cells/well) and incubated at 37 °C under 5% CO_2 for 24 h. Cultured media were replaced with **I₂-IR783/MTX@NPs** containing 5 μM **I₂-IR783** for 24 h. Then, the cells were washed with PBS (3 times) before being incubated with either singlet oxygen sensor green (SOSG, 20 mM, Thermo Fisher Scientific) or 2,7-dichloro-dihydro-fluorescein diacetate (DCFH-DA, 20 mM, Sigma-Aldrich) for 2 h. After washing with PBS, the cells were irradiated by an 805 nm red LED lamp at a power density of 18 mW cm^{-2} for 30 min and re-incubated for 20 min. Non-irradiated cells were used as a control. Before imaging, the cells were washed with PBS (3 times) followed by nucleus staining with 1.0 μM Hoechst 33342. The cells were visualized by LSCM with a 60X oil immersion objective lens (Nikon A1Rsi). A 488 nm excitation laser (Em: 500-550 nm) was used to detect the fluorescence products of DCFH-DA, 2,7-dichloro-dihydro-fluorescein (DCF), and SOSG, while Hoechst 33342 was excited at 408 nm (Em: 425-475 nm).

3.3.10 3D-models formation

Spheroids of 4T1 cells were generated in a 96-well cell carrier spheroid Ultra Low Attachment (ULA) microplate (PerkinElmer). Briefly, 2.5×10^3 4T1 cells per well were seeded in RPMI-1640 supplemented with 10% FBS. Then, the 4T1 spheroids were grown in the incubator for 3 days at 37 °C under 5% CO_2 for future use.

3.3.11 3D Live-cell imaging

The localization of free dye and NPs was evaluated in 4T1 spheroids exposed to the drug for 24 h by removing 50 μL of media and replacing it with 50 μL of the 2X solutions of PSs in complete medium at various time points (0, 3, 12, and 24 h). After that, the spheroid cells were then stained with a 2X concentration of

Hoechst 33342 in media to final concentrations of 20 $\mu\text{g/mL}$ for 30 min. The spheroids were imaged using a LSCM with a 10X objective lens.

3.3.12 3D LIVE/DEAD viability/cytotoxicity assay

Three-day-old spheroids of 4T1 were incubated with the solutions of PSs in complete medium with final concentrations of 10 μM . After 24 h incubation, the cells were irradiated by NIR light before re-incubation for another 24 h. The spheroid cells were stained with a 2X staining solution in media prepared by mixing calcein AM and propidium iodide (PI) (Thermo Fisher Scientific) to final concentrations of 2 μM and 8 $\mu\text{g/mL}$, respectively. The plates were then incubated at 37 °C under 5% CO_2 for 30 min and then imaged by LSCM with a 10X objective lens with 488 nm excitation for calcein AM (Em: 500–550 nm) and 561 nm excitation for PI (Em: 600–650 nm).

3.3.13 Frequency Domain Optical Coherence Tomography (FD-OCT)

The 3D imaging by FD-OCT was conducted in collaboration with Assoc. Prof. Dr. Panomsak Meemon's group. Optical coherence tomography (OCT) is a non-contact and non-destructive imaging technique that constructs cross-sectional images of biological tissues by utilizing the low-coherence property of broadband near-infrared light to detect both the amplitude and depth of the backscattered light from the tissue (Aumann, Donner, Fischer, and Müller, 2019).

In this study, an in-house developed FD-OCT system was used to perform 3D imaging of the cultured 4T1 spheroid cells. The FD-OCT imaging system employed a broadband light source at a wavelength range between 800 nm and 880 nm, centered around 840 nm, and had an illumination power with less than 3 mW. The depth resolution and horizontal resolution of the FD-OCT imaging system were approximately 10 μm and 4 μm , respectively. The depth penetration of the developed OCT prototype was up to 2 mm from the sample's surface. The captured 3D datasets were processed and visualized with ImageJ software (NIH, USA).

3.4 Results and discussion

3.4.1 Preparation and characterization of I₂-IR783/MTX@NPs

The nanoparticles (NPs) were prepared by a modified nanoprecipitation method (Hu et al., 2020; Yosi Shamay et al., 2018). MTX in DMSO solution was gradually added to an aqueous solution of I₂-IR783 while gently vortexing. The resulting I₂-IR783/MTX@NPs were purified via centrifugation and then suspended in phosphate-buffered saline (PBS, pH = 7.4), as shown in **Figure 3.2A**. **Figure 3.2B** illustrates that the absorption spectra of free I₂-IR783 and the nanoparticles were nearly identical, indicating that I₂-IR783 was successfully incorporated into the nanoparticles. Similarly, the absorption spectra of free MTX and the nanoparticles in the 300–450 nm range displayed strong similarities as shown in supporting information, confirming the presence of MTX in the nanoparticles. Furthermore, when free I₂-IR783 dye and NPs in PBS were compared, they showed different visual appearances. The free dye I₂-IR783 formed a greenish-blue solution (vial i, **Figure 3.2B**), while the nanoparticles produced a vibrant yellow-green solution (vial ii, **Figure 3.2B**).

Fourier transform infrared (FTIR) spectroscopy analysis of free MTX, I₂-IR783, and I₂-IR783/MTX@NPs (**Figure 3.2C**) confirmed the successful formation of I₂-IR783/MTX nanoparticles. In the FTIR spectrum of free MTX, the peak around 3500–3400 cm⁻¹ indicated the presence of an NH group, and the carboxylic group was reflected by the transmittance at 3001 and 2916 cm⁻¹, along with the C=O stretching at 1657 cm⁻¹. The C=C vibration in the aromatic ring and the C–N stretching in the aromatic amine contributed to the peaks in the 1500–1200 cm⁻¹ region. For I₂-IR783, the S=O stretching of sulfate groups was clearly evident in the 1400–1300 cm⁻¹ region. The FTIR spectrum of I₂-IR783/MTX@NPs showed peaks similar to those of free MTX and I₂-IR783, confirming the successful self-assembly of MTX and I₂-IR783. Finally, the concentrations of MTX and I₂-IR783 in the nanoparticles were determined by a UV–vis–NIR spectrophotometry.

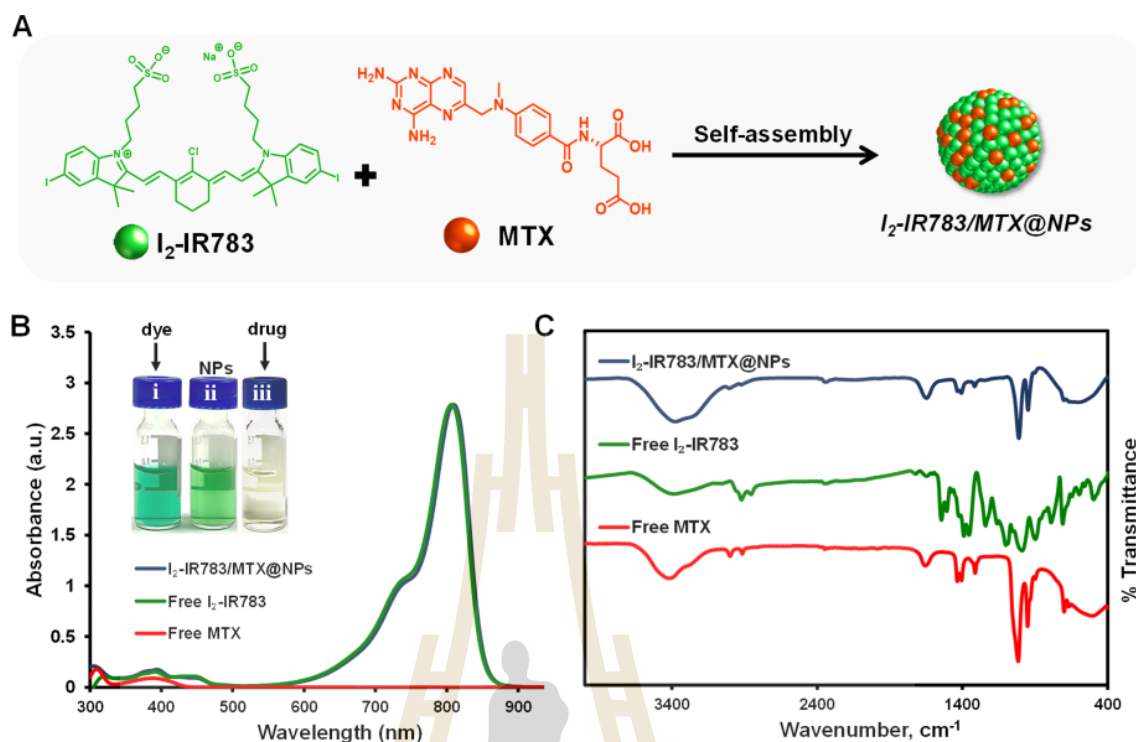


Figure 3.2 (A) The scheme illustrates I_2 -IR783 self-assembled with MTX to form nanoparticles for PDT and chemotherapy. (B) Absorption spectra of NPs, free dye, and free MTX; the vials contain free I_2 -IR783 (i), I_2 -IR783/MTX@NPs (ii), and free MTX (iii). (C) FTIR of free MTX, I_2 -IR783, and I_2 -IR783/MTX@NPs.

The size of the nanoparticles (NPs) was determined by dynamic light scattering (DLS), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). In PBS (pH = 7.4), the hydrodynamic diameter of the NPs was measured to be 240.6 ± 2.5 nm, with an excellent polydispersity index (PDI) of 0.170 ± 0.007 and a negative surface charge of -6.26 ± 2.82 mV, as evaluated by DLS (Figure 3.3B). TEM and SEM images (Figures 3.3C and 3.3D) revealed uniform particle size, which was consistent with the DLS size distribution in PBS. Furthermore, the NPs were found to be stable in both PBS and serum but disaggregated in fetal bovine serum (FBS) (Table 3.1). All of these results confirmed that MTX successfully self-assembled with I_2 -IR783 to form I_2 -IR783/MTX@NPs.

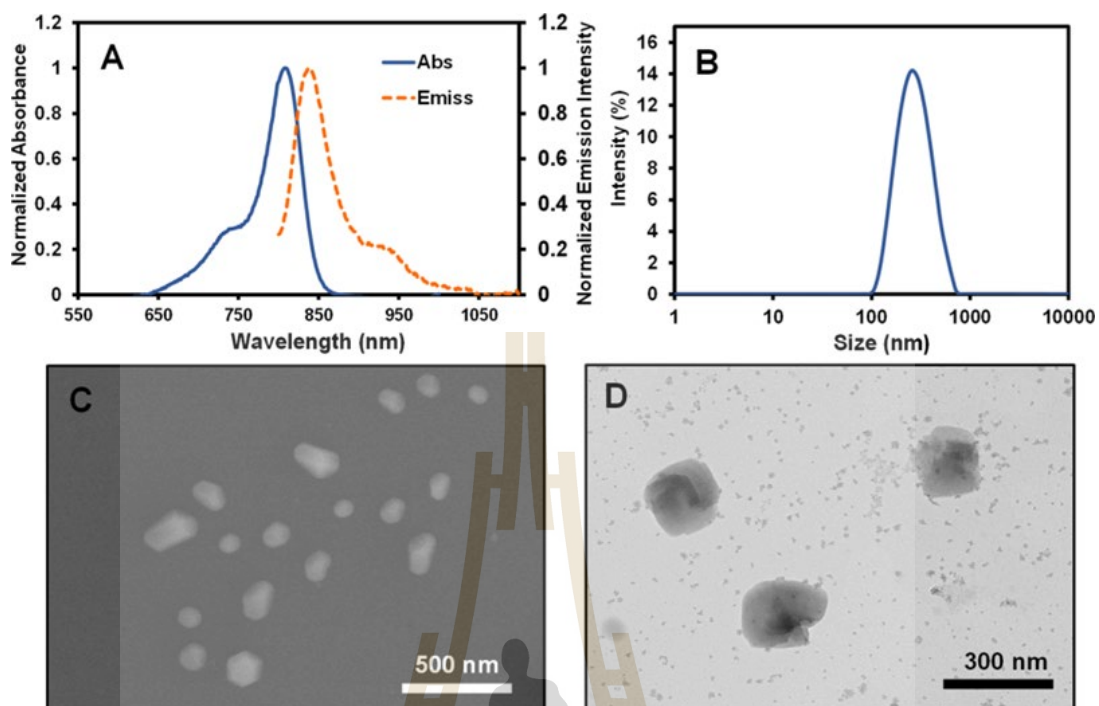


Figure 3.3 (A) Vis-NIR absorption and emission spectra of I_2 -IR783/MTX@NPs in PBS (3% Tween 80). (B) The size distribution of the I_2 -IR783/MTX@NPs was analyzed by DLS, (C) SEM, and (D) TEM. Scale bar = 500 nm.

Table 3.1 Characteristics of I_2 -IR783/MTX@NPs in DI water, PBS at pH 5.0, PBS at pH 7.4, RPMI and FBS by DLS and zeta potential measurements.

Entry	Medium	DLS size (nm) (n = 3)	PDI (n = 3)	Zeta potential (mV) (n = 3)
1	DI water	242.1 ± 5.4	0.251 ± 0.041	-6.60 ± 1.04
2	PBS at pH 5.0	287.5 ± 14.9	0.442 ± 0.021	-4.82 ± 0.52
3	PBS at pH 7.4	240.6 ± 7.4	0.170 ± 0.007	-6.26 ± 2.82
4	RPMI	292.3 ± 11.3	0.208 ± 0.021	-0.76 ± 0.04
5	FBS	102.2 ± 6.14	0.126 ± 0.005	-14.4 ± 0.71

3.4.2 Photophysical properties of I₂-IR783/MTX@NPs

In **Figure 3.4**, the absorbance and fluorescence spectra of I₂-IR783/MTX@NPs were measured in PBS with 3% Tween 80. The nanoparticles exhibited absorption and emission in the near-infrared range, with absorption maxima at 808 nm and emission maxima at 838 nm, and a fluorescence quantum yield of 0.167, compared to ICG ($\Phi_f = 0.13$). Additionally, 1,3-diphenylisobenzofuran (DPBF), a singlet oxygen (¹O₂) scavenger, was used to assess the ability of I₂-IR783/MTX@NPs and free I₂-IR783 to generate singlet oxygen upon activation by NIR 805 LED lamp. Singlet oxygen production was observed after irradiating I₂-IR783/MTX@NPs and free I₂-IR783 with NIR light at a power density of 18 mW cm⁻² for 10 minutes, indicated by the decrease in DPBF absorbance at 408 nm over time (Figure 3.3). Compared to indocyanine green (ICG, the standard photosensitizer, $\Phi_\Delta = 0.077$), the calculated singlet oxygen quantum yield (Φ_Δ) was significantly improved for both I₂-IR783/MTX@NPs (4.7-fold, $\Phi_\Delta = 0.36$) and free I₂-IR783 (4.5-fold, $\Phi_\Delta = 0.35$). This suggested that the formation of nanoparticles did not affect the ¹O₂ production by I₂-IR783, implying that the NPs could be effectively used in photodynamic therapy (PDT).

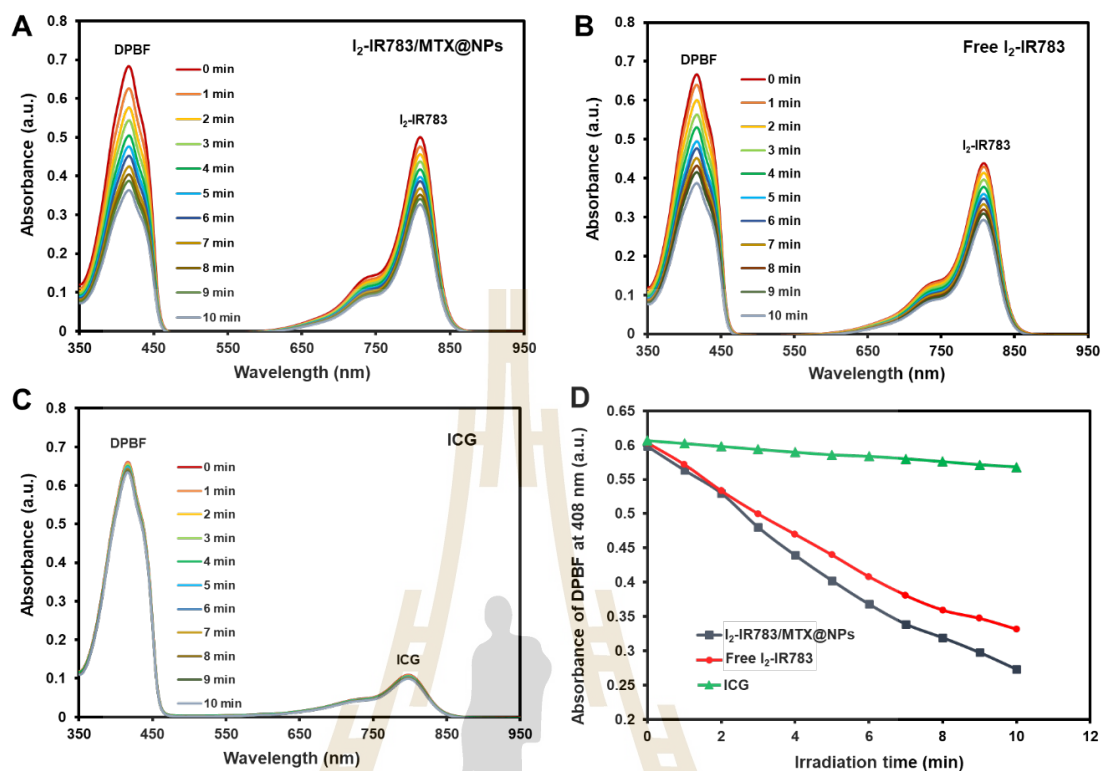


Figure 3.4 Reduction in UV-Vis-NIR absorbance of the DPBF solution with (A) I₂-IR783/MTX@NPs containing 2 μ M I₂-IR783, (B) free I₂-IR783 (2 μ M), and (C) ICG (2 μ M) in PBS (3% Tween 80). (D) time-dependent UV-Vis-NIR absorbance change of DPBF with I₂-IR783/MTX@NPs containing 2 μ M I₂-IR783 and free I₂-IR783 (2 μ M) compared to standard (ICG, 2 μ M) under light irradiation by NIR 805 LED lamp in PBS (3% Tween 80) for 10 min (1 min intervals).

3.4.3 Cell internalization of I₂-IR783/MTX@NPs

To evaluate the ability of the nanoparticles (NPs), the cellular uptake of I₂-IR783/MTX@NPs was studied in murine breast cancer (4T1) cells and human foreskin fibroblast (HFF) cells, representing cancerous and normal cells, respectively. Internalization assays were performed in 4T1 cells to evaluate the accumulation of I₂-IR783/MTX@NPs (containing 5 μ M I₂-IR783) over various time intervals. As observed in the confocal images (Figure 3.5A), I₂-IR783/MTX@NPs were successfully internalized and accumulated within the 4T1 cells, with a notable increase in

accumulation within the first 6 hours of incubation. However, I_2 -IR783/MTX@NPs demonstrated a significantly higher uptake in cancer cells compared to normal cells (Figure 3.5B).

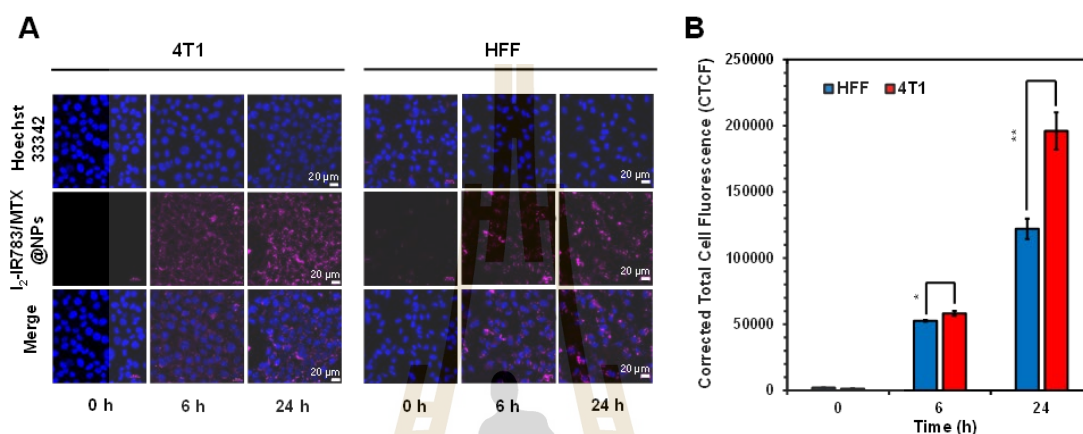


Figure 3.5 (A) Confocal images of 4T1 and HFF cells incubated with I_2 -IR783/MTX@NPs containing I_2 -IR783 5 μ M for various time points (0, 6, and 24 h); I_2 -IR783/MTX@NPs fluorescent signal was displayed in magenta, DAPI nucleus co-staining signal was shown in blue. Scale bars = 20 μ m. (B) Quantitative fluorescent intensity is represented as corrected total cell fluorescence (CTCF), which was quantified using ImageJ and represents the mean $\pm$ SD ($n=3$, 30 cells/set). Statistical analysis is based on a T-test (* $P < 0.05$, ** $P < 0.01$).

3.4.4 Photodynamic therapy

The photocytotoxicity of I_2 -IR783/MTX@NPs was assessed to confirm the combined effectiveness of photodynamic therapy (PDT) and chemotherapy. In the cell viability tests, 4T1 and HFF cells were treated with different concentrations (0–20 μ M) of I_2 -IR783/MTX@NPs, free I_2 -IR783, and free MTX for 24 hours. After treatment, the cells were exposed to an 805 nm LED lamp for 30 minutes, and cell viability was measured under both dark and light conditions using the MTT assay (Van Meerloo et al., 2011).

In this study, sufficient singlet oxygen was generated by exposing the cells to an 805 nm LED lamp at a power density of 18 mW/cm² for 30 min (equivalent to approximately 32.4 J/cm²). In the absence of light, free I₂-IR783 displayed low toxicity to both cancer and normal cells (**Figure 3.6A, D**). However, after light exposure, there was a significant reduction in cell viability, especially in cancer cells (**Figure 3.6A**). Interestingly, MTX showed higher toxicity in cancer cells compared to normal cells, regardless of light exposure (**Figure 3.6B, E**), with 4T1 cells exhibiting strong resistance to MTX treatment (**Figure 3.6B**). On the other hand, I₂-IR783/MTX@NPs caused a marked decrease in cell viability, particularly in 4T1 cells under NIR light exposure (**Figure 3.6C**). Remarkably, I₂-IR783/MTX@NPs were more effective at reducing cell viability than MTX alone at a concentration of 10 μM, overcoming MTX resistance. In contrast, HFF cells showed minimal cytotoxicity even at 20 μM, with their viability remaining around 50% under NIR light exposure (**Figure 3.6F**). Moreover, when comparing the cell viability of 4T1 cells treated with NPs to those treated with free dye or free drug at the same concentration and under light conditions, the NPs exhibited the most significant cytotoxicity.



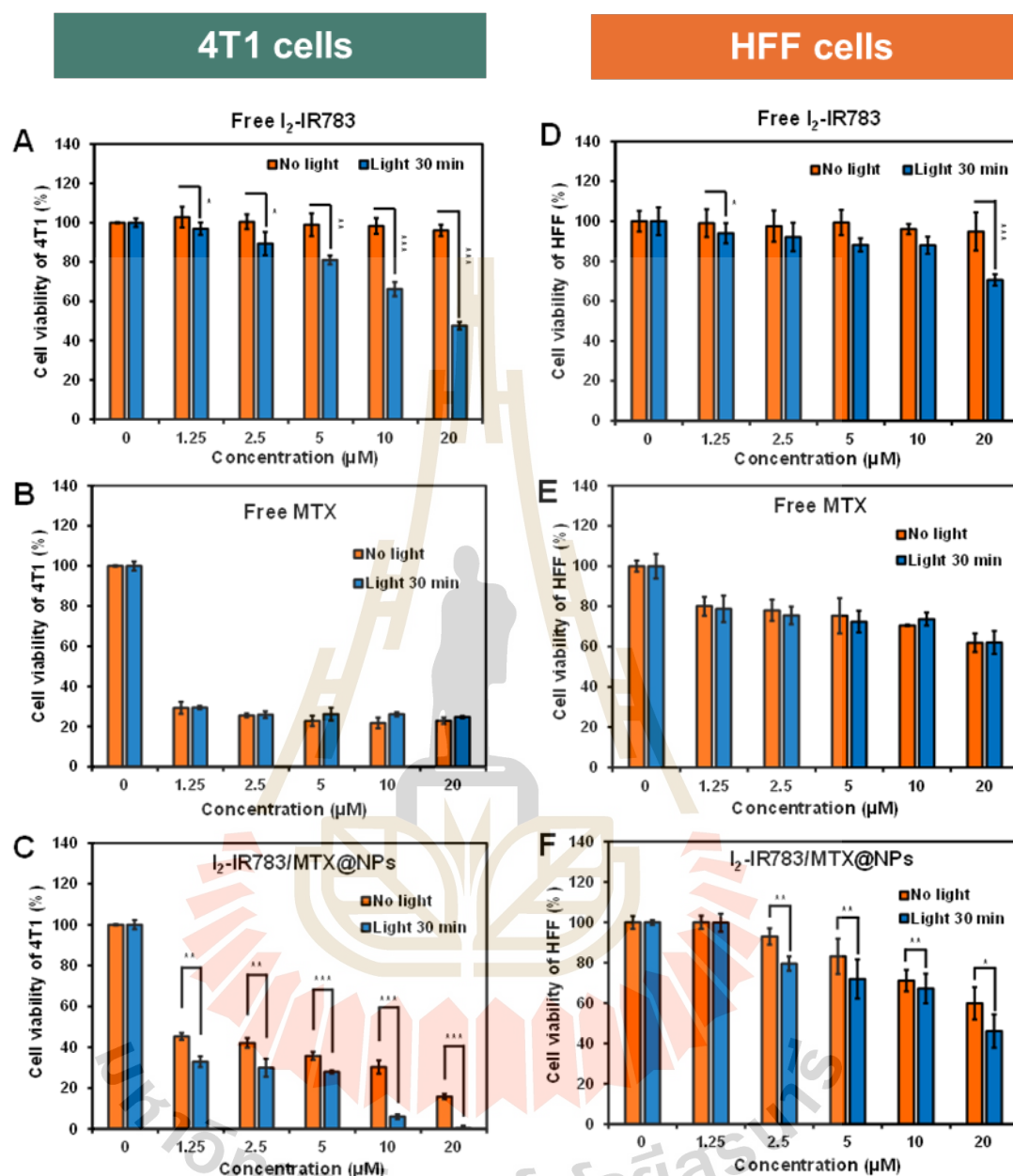


Figure 3.6 Relative of cell viability of 4T1 and HFF cells under irradiation (0 or 30 min) after incubation with Free I₂-IR783 (A&D), free MTX (B&E), and I₂-IR783/MTX@NPs (C&F) containing free dye I₂-IR783 (0-20 μM) for 24 h; blue bars represent cells irradiated with an 805 lamp for 30 min and orange bars represent cells without irradiation. Data are presented as mean ± SD (n = 4), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 are based on Student's T-test.

To confirm the synergistic action of the drug and photosensitizer, 4T1 cells were also treated with combinations of free I_2 -IR783 and free MTX mixtures followed by light exposure. Since cell viability remained the same at all concentrations, no synergistic effect was observed in this case (Figure 3.7). These findings clearly indicated that I_2 -IR783/MTX@NPs are biocompatible as drug delivery vehicles and show a strong synergistic therapeutic effect when combined with PDT and chemotherapy. Therefore, I_2 -IR783/MTX@NPs hold potential as a cancer-targeted theranostic agent for breast cancer treatment.

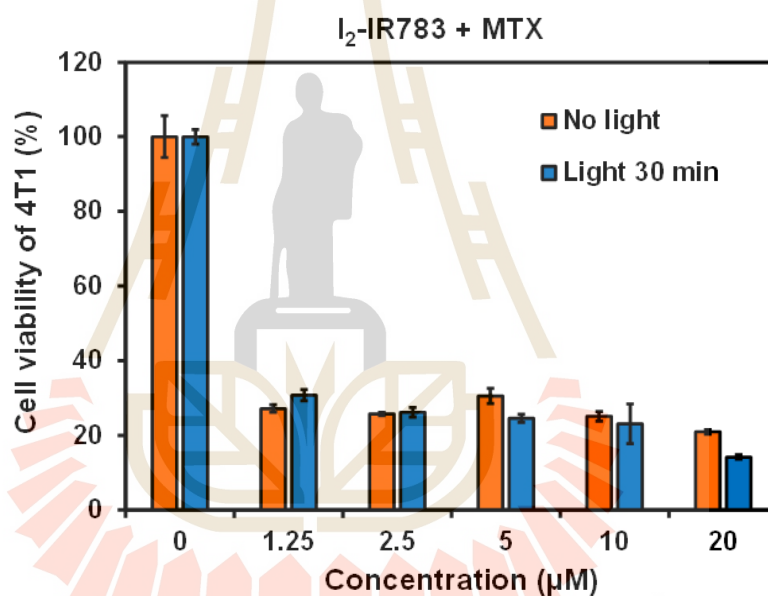


Figure 3.7 Relative of cell viability of 4T1 under irradiation (0 or 30 min) after incubation with free dye I_2 -IR783 and MTX mixtures (0-20 μM) for 24 h; blue bars represent cells irradiated with an 805 lamp for 30 min and orange bars represent cells under dark conditions.

3.4.5 Intracellular reactive oxygen species (ROS) and $^1\text{O}_2$ generations

Next, we assessed ROS generation using 2,7-dichloro-dihydro-fluorescein diacetate (DCFH-DA) to detect ROS in live cells, as it is oxidized by ROS into the fluorescent compound 2,7-dichlorofluorescein diacetate (DCF) (Aranda et al., 2013). DCF emits green fluorescence when excited at 488 nm, which can be observed under a confocal microscope. 4T1 cells were treated with $\text{I}_2\text{-IR783/MTX@NPs}$ for 24 hours, then stained with DCFH-DA, and exposed to an 805 nm LED lamp for 30 minutes. As indicated in **Figure 3.8A**, only the cells that carried NPs and were exposed to light showed intense green DCF fluorescence, suggesting ROS production inside the cells. In contrast, no ROS production was detected in the control groups (cells alone, cells with DCFH-DA, and cells with $\text{I}_2\text{-IR783/MTX@NPs}$, rows 1, 2, and 4, respectively). Additionally, to monitor singlet oxygen ($^1\text{O}_2$) production, we used singlet-oxygen sensor green (SOSG) as a probe (Gollmer et al., 2011). Green fluorescence was observed only in cells treated with $\text{I}_2\text{-IR783/MTX@NPs}$ and exposed to light (**Figure 3.8A**, row 6), while none of the control groups showed fluorescence (columns 1, 3, and 4). In the absence of light irradiation (**Figure 3.8B**), no significant green fluorescence from DCF or SOSG was detected in any group. These findings provide strong evidence that ROS, including $^1\text{O}_2$, were generated and caused damage to cancer cells upon light activation of $\text{I}_2\text{-IR783/MTX@NPs}$.

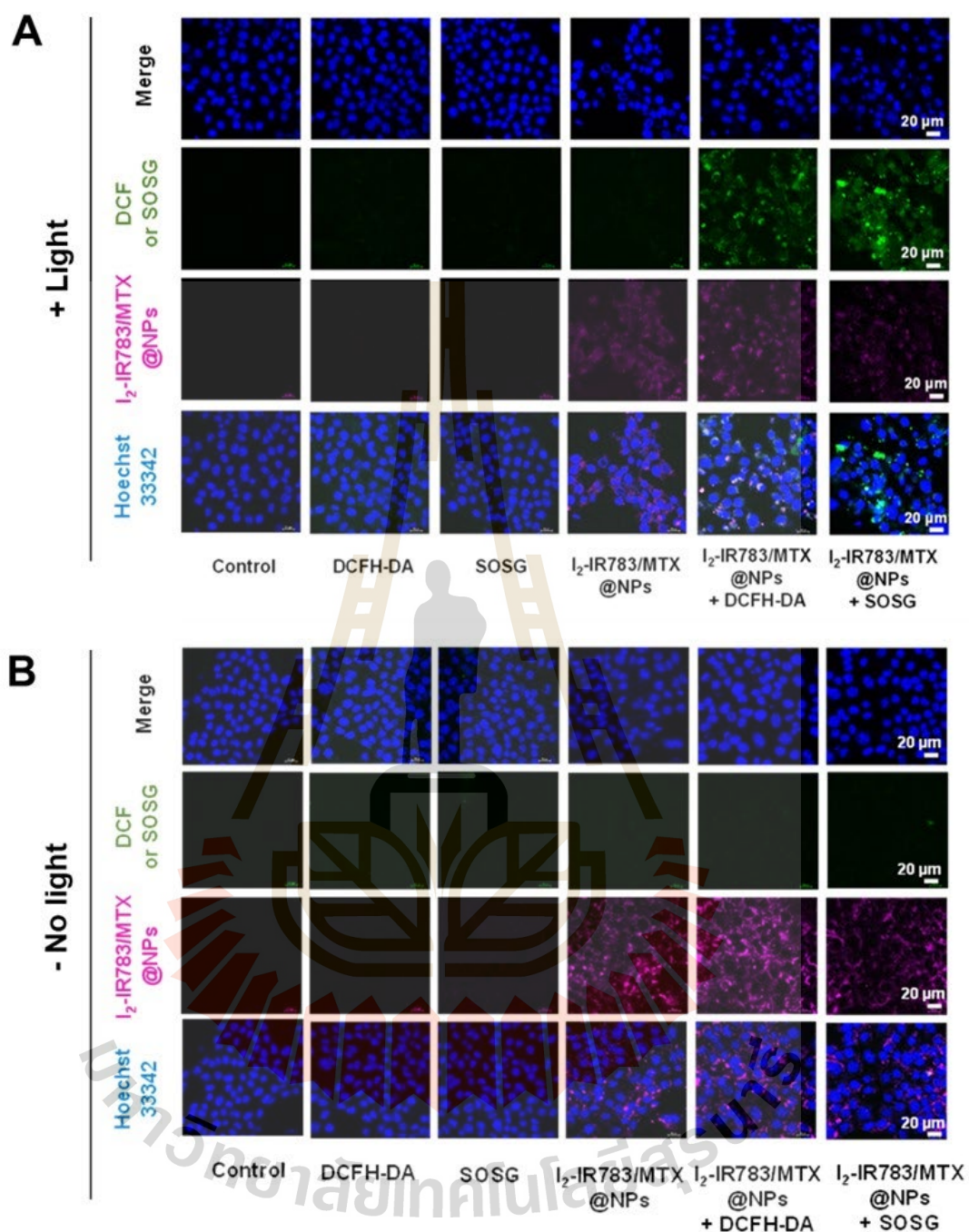


Figure 3.8 Intracellular ROS detection generated by I_2 -IR783/MTX@NPs (5 μ M) in 4T1 cells by using DCFH-DA and SOSG upon (A) light irradiations and (B) dark conditions; non-treated cells (1st row); DCFH-DA (2nd row); SOSG (3rd row); I_2 -IR783/MTX@NPs (4th row); I_2 -IR783/MTX@NPs and DCFH-DA (5th row); I_2 -IR783/MTX@NPs and SOSG (last row). Scale bar = 20 μ m.

3.4.6 *In vitro* studies on three-dimensional (3D) breast tumor models

Cells grown in 3D culture models more closely resemble the complex conditions found *in vivo* (Vinci et al., 2012). As a result, research using 3D culture models tends to offer more accurate insights for *in vivo* applications. Therefore, the anticancer activity of I_2 -IR783/MTX@NPs was also assessed in 3D tumor models.

Confocal microscopy was employed to study the internalization of nanoparticles in tumor spheroids at different incubation times (0, 3, 12, and 24 h) to identify the optimal timing for PDT therapy administration. As shown in **Figure 3.9**, the degree of NP internalization in 4T1 spheroids increased with longer incubation times, with the strongest fluorescence observed at 24 hours. This pattern was similar to the accumulation of free I_2 -IR783 within the spheroid structure. These results are in line with findings from cellular uptake studies in two-dimensional (2D) cell cultures.

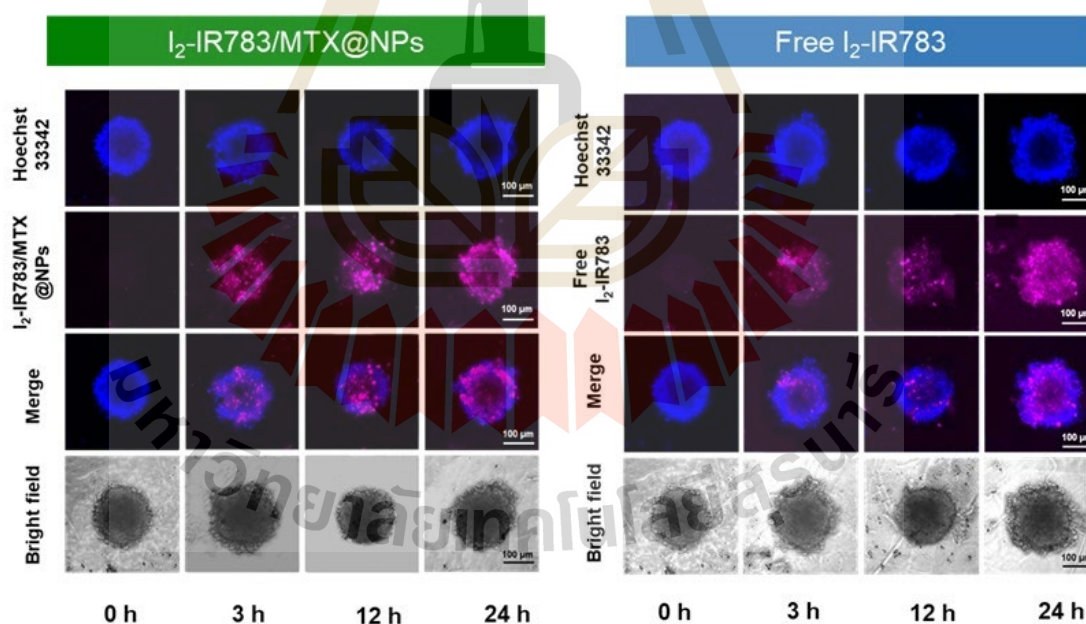


Figure 3.9 Confocal images of 4T1 spheroids incubated with I_2 -IR783/MTX@NPs (left; [free I_2 -IR783] and [MTX] = 5 μ M) and free I_2 -IR783 (right) for various time points (0, 3, 12, and 24 h). Scale bars = 100 μ m.

To evaluate the effectiveness of I_2 -IR783/MTX@NPs compared to free I_2 -IR783 and free MTX, we performed photocytotoxicity assays under both dark and light conditions in 3D in vitro models, using the same incubation time and irradiation method applied to 2D cell cultures. The LIVE/DEAD viability/cytotoxicity assay was used to assess cell viability. After treating 4T1 spheroids with NPs, free dye, or free drug, followed by light exposure, the spheroids were stained with calcein AM and propidium iodide (PI) to visualize live and dead cells, respectively. Calcein AM is taken up by live cells and emits green fluorescence when excited with a 495 nm laser. In contrast, PI enters dead cells, binds to their DNA, and emits red fluorescence when excited with a 535 nm laser. The results revealed that red fluorescence was mostly observed at the outer edges of the spheroids in all groups (Figure 3.10). When the spheroids containing NPs were exposed to light, there was a significant increase in dead cells, while MTX treatment induced red fluorescence in both light-exposed and non-irradiated cells.

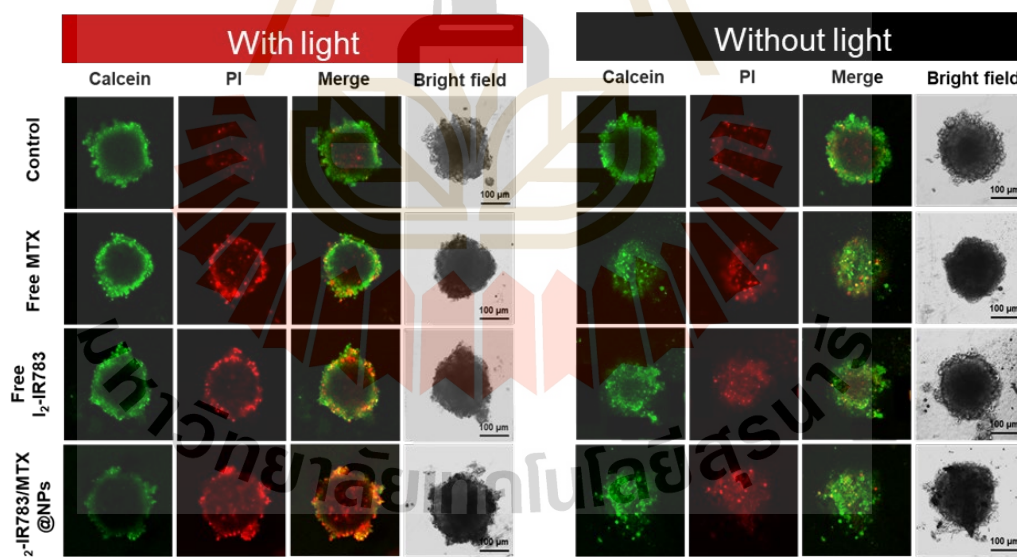


Figure 3.10 LIVE/DEAD viability/cytotoxicity assay of 4T1 spheroid cells incubated with free MTX (10 μ M), free I_2 -IR783 (10 μ M), and I_2 -IR783/MTX@NPs ($[I_2$ -IR783] and [MTX] = 10 μ M) under dark and light conditions; non-treated cells (1st row); free MTX (2nd row); free I_2 -IR783 (3rd row); I_2 -IR783/MTX@NPs (last row). Scale bars = 100 μ m.

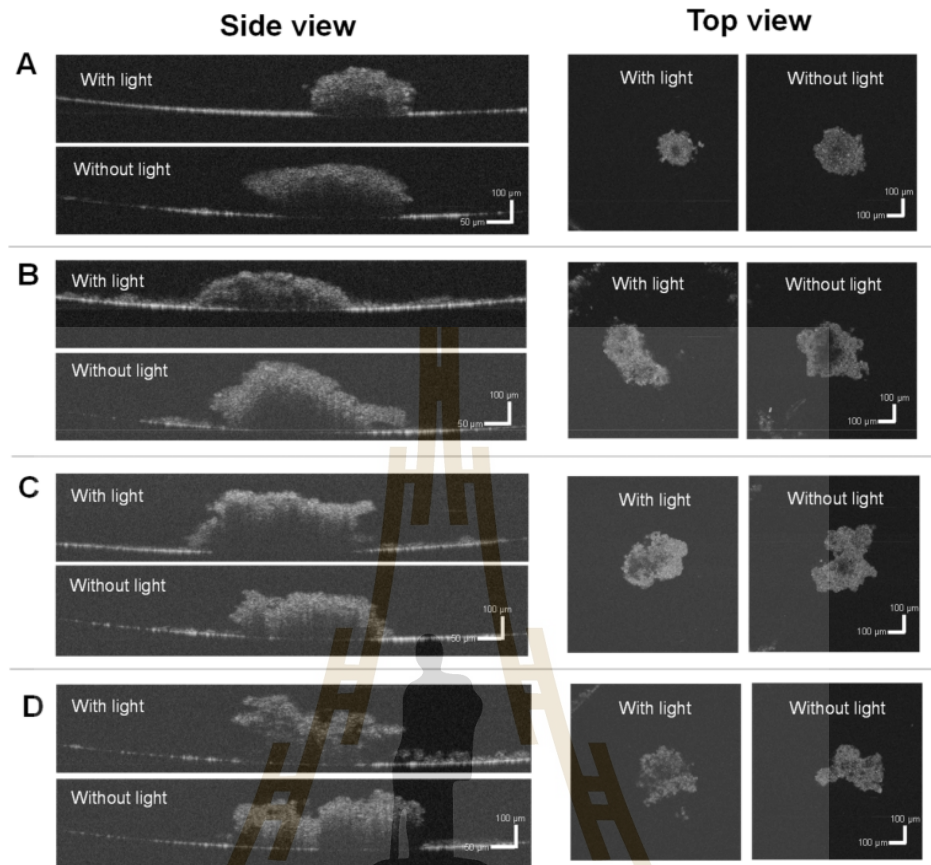


Figure 3.11 3D cell imaging using OCT in 4T1 spheroid cells under dark and light conditions; (A) non-treated cells, (B) free MTX (5 μM), (C) free I₂-IR78 (5 μM), and (D) I₂-IR783/MTX@NPs ([I₂-IR783] and [MTX] = 5 μM). Scale bars = 100 μm.

Finally, optical coherence tomography (OCT) was utilized to observe the therapeutic response following PDT in tumor spheroids. OCT is a non-invasive imaging method that allows for high-speed, high-resolution, depth-cross-sectional imaging of biological tissues (Aumann et al., 2019; Vakoc, Fukumura, Jain, and Bouma, 2012). It has been applied in monitoring tumor progression during cancer treatment, helping to assess tumor size, visualize the vascular structure (Snuderl et al., 2013), monitor lymphangiogenesis and lymphatic dysfunction, and differentiate between viable and non-viable areas of the tumor (Nam, Vakoc, Blauvelt, and Chico-Calero, 2015). **Figure 3.11A** presents OCT images of untreated spheroids under both dark and light conditions, where the tumor retains a well-formed spherical shape in both cases.

However, after 24 hours of treatment with the chemotherapy drug MTX, some damage to the spheroid was observed under both conditions (**Figure 3.11B**). In contrast, treatment with **I₂-IR783** caused damage only under light exposure (**Figure 3.11C**). Significant damage was seen when the spheroid was treated with **I₂-IR783/MTX@NPs** followed by light exposure (**Figure 3.11D**). These results provide clear evidence of the synergistic anticancer effect of our nanoparticles.

3.5 Conclusion

In conclusion, stable nanoparticles designed for a combination of photodynamic therapy (PDT) and chemotherapy were successfully fabricated by self-assembling the cyanine dye (**I₂-IR783**) and the chemotherapeutic agent (MTX). The resulting nanoparticles exhibited absorption and emission spectra within the Vis-NIR range, making them ideal for both PDT and fluorescence imaging applications. Upon exposure to 805 nm LED light for 30 minutes, the **I₂-IR783/MTX@NPs** produced a sufficient amount of singlet oxygen (¹O₂), which was effective in killing cancer cells. The **I₂-IR783/MTX@NPs** demonstrated significant light-triggered cytotoxicity to 4T1 cells at concentrations as low as 10 μ M, reducing cell viability to under 10% in physiological conditions. In contrast, cells treated with MTX alone showed substantial resistance, maintaining around 30% viability even at 20 μ M. Moreover, the **I₂-IR783/MTX@NPs** preserved their PDT and chemotherapy efficacy in 3D tumor spheroid models. Finally, optical coherence tomography (OCT), a non-invasive imaging technique, was used to monitor the progress of the treatment. Overall, the results confirmed that the combination of **I₂-IR783** and MTX displayed synergistic anticancer effects through a combined photo-chemotherapy approach in both 2D and 3D cell cultures. Therefore, **I₂-IR783/MTX@NPs** present a promising potential as a carrier-free nanomedicine for synergistic photo-chemotherapy.

3.6 References

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CHAPTER IV

HEAVY-ATOM-FREE AZA-BODIPY POLYMERIC NANOPARTICLES FOR SUPERIOR IMAGING-GUIDED PHOTODYNAMIC THERAPY

4.1 Abstract

Photodynamic therapy (PDT) stands out as a promising modality for cancer treatment due to its non-invasive nature and high specificity. Nonetheless, the majority of photosensitizers (PSs) exhibiting photodynamic traits contain heavy atoms, leading to cellular toxicity concerns. The quest for advanced photosensitizers suitable for the clinical translation of PDT remains a challenge. This study introduces a heavy-atom-free aza-BODIPY (**AB-OH**) compound featuring a donor-acceptor-donor (D-A-D) structure, coupled with poly(ethylene glycol)-succinic acid polymer (mPEG-SA). Upon self-assembly into nanoparticles, **AB-OH@mPEG-SA** demonstrated the ability to generate reactive oxygen species (ROS) and heat when exposed to 660 nm red LED lamp irradiation. Assessments in 4T1 breast cancer cell treatment validated the low dark toxicity and remarkable photocytotoxicity of **AB-OH@mPEG-SA** NPs through intracellular ROS detection and live/dead staining assays. In addition, **AB-OH@mPEG-SA** displayed light-induced cytotoxicity in 4T1 cells, whereas the non-irradiated cells maintained full viability. Furthermore, the NPs demonstrated superior anticancer activity compared to the parent **AB-OH** compound. This study underscores the potential of **AB-OH@mPEG-SA** NPs, exhibiting promising PDT/mild PTT (photothermal therapy) efficacy, as prospective candidates for cancer diagnosis and treatment.

4.2 Introduction

Aza-BODIPY derivatives have emerged as highly promising photosensitizers (PSs) for photodynamic therapy (PDT) due to their exceptional properties, including high fluorescence quantum yields (with minimized nonradiative decay), tunable absorption and emission spectra ranging from visible to near-infrared light, as well as

impressive photostability and chemical stability (Chen et al., 2018; Tian et al., 2022; Treekoon et al., 2021; Wu et al., 2019; Yu et al., 2020; M. Zhao et al., 2018). In comparison to other organic photosensitizers, such as porphyrins, cyanines, and squaraines, aza-BODIPY derivatives show superior photostability, which enhances their biosafety and therapeutic effectiveness in phototherapy (Cai et al., 2018).

Additionally, compared to some organic photosensitizers such as porphyrins, cyanines, and squaraines, they exhibit superior photostability, allowing for improved biosafety and therapeutic efficacy during phototherapy (Cai et al., 2018). Although heavy atoms are typically included in the development of aza-BODIPY-based PSs with PDT characteristics to effectively facilitate the intersystem crossing (ISC) and subsequently generate ROS, they still have certain disadvantages, including increased dark toxicity, short triplet state lifetimes, and low photostability, which inadvertently impede their use in clinical and biomedical environments. As a result, it is critical to explore innovative aza-BODIPY PSs that do not involve heavy atoms to evaluate their potential clinical uses (J. Zhao, Xu, Yang, Wang, and Zhong, 2015).

Furthermore, optimization of tumor-targeting ability is a key factor for tumor therapy. Nanotechnology has played an important role in cancer research (Bertrand, Wu, Xu, Kamaly, and Farokhzad, 2014; Lim, Lee, Zhang, and Chu, 2012; Peer et al., 2007). Most of the existing PS-based NPs have been created by the noncovalent assembly of dye molecules and a polymeric matrix. (Yadav, Sharma, and Kumar, 2020) However, there are some limitations of polymeric encapsulated NPs, such as burst release, poor drug loading, large size distribution, and limits on long-term storage (Banik, Fattahi, and Brown, 2016; Singh, Biswas, Shukla, and Maiti, 2019). Although the covalently bonded polymer-PS system has received less attention, it has some intriguing qualities that could make it a viable cancer treatment option, such as regioselective synthesis, scalability, straightforward formulation, high drug loading, and long-term storage (Canal, Sanchis, and Vicent, 2011; Kopeček, 2013).

In this study, we have synthesized a new heavy-atom-free aza-BODIPY analog enriched with methoxy, and hydroxyl groups (referred to as **AB-OH**) intended for diagnostic and phototherapeutic purposes. To enhance the compound's ability to target tumors, the developed PS was covalently linked with poly(ethylene glycol)-

succinic acid polymer (mPEG-SA), resulting in the self-assembly of a stable nanoparticle (AB-OH@mPEG-SA). Following nanoformulation, these NPs underwent a comprehensive examination of their chemical, physical, and optical characteristics to assess their potential utility in cancer imaging. Both *in vitro* and *in vivo* tests were conducted to evaluate this nanosystem, indicating that **Figure 4.1** outlines the overall concept underlying this research.

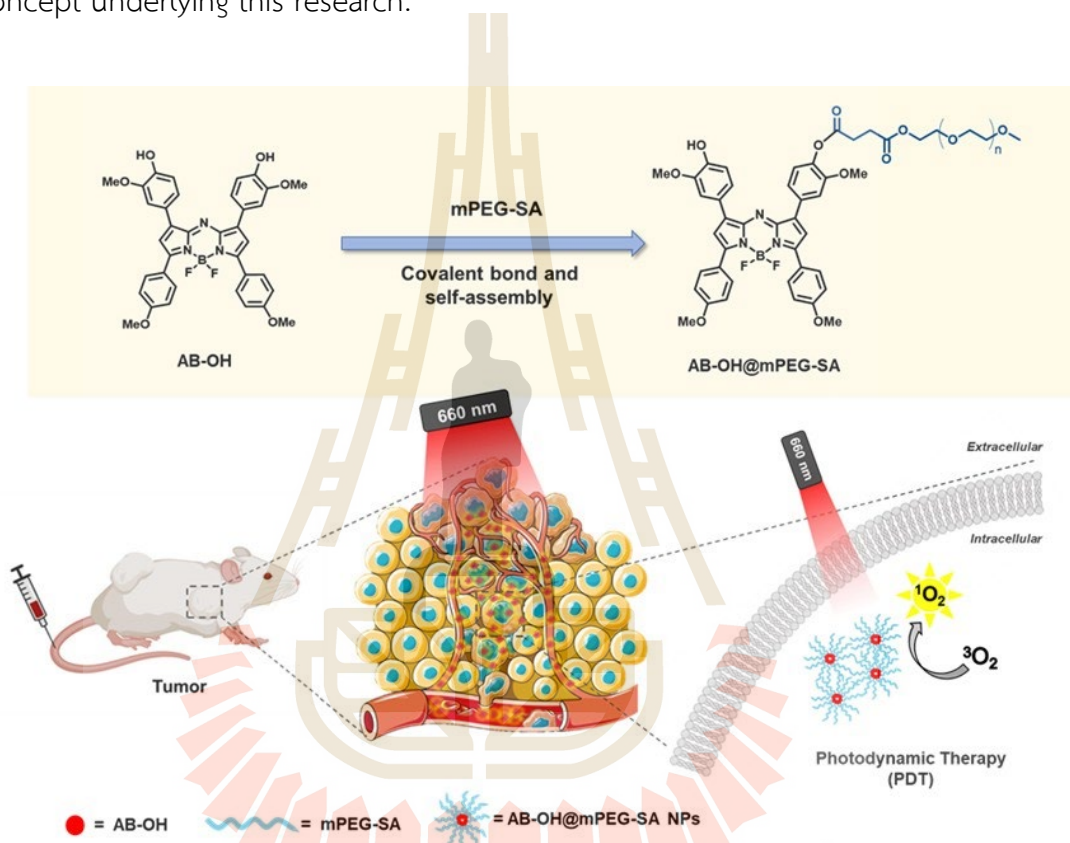


Figure 4.1 The overall concept of the aza-BODIPY-based polymeric nanoparticles for photodynamic cancer therapy in animal models.

4.3 Experimental

4.3.1 Materials and instruments

All reagents were purchased from Aldrich and TCI and used without further purification. All column chromatography was performed with the use of Merck Silica gel (230-400 mesh). The nuclear magnetic resonance (NMR) spectra were recorded on a 500 MHz (^1H) and 125 MHz (^{13}C) NMR spectrometer (Avance III,

Spectrometer, Bruker Biospin Ag, USA). A Bruker Microflex MALDI-TOF mass spectrometer was used to obtain the high-resolution mass spectra of the samples. A Cary Series UV-Vis-NIR spectrophotometer was used to collect UV-vis-NIR absorption spectrum data (Agilent Tech). Emission spectra were recorded using a PTI QuantaMaster 500 Near Infrared Photoluminescence System (HORIBA Scientific). Dynamic light scattering (DLS) measurement was measured by the Zetasizer Nano ZS (Malvern Panalytical). Images from a JEM-2100-Plus Transmission Electron Microscope were taken using a transmission electron microscope (TEM). DLS and TEM were used to determine the size of NPs. Fourier transform infrared spectrophotometer (FTIR, PerkinElmer Spectrum GX) was utilized to collect IR spectra. To confirm the 3D structure of the cultured cells, 3D microscopic imaging of the tumor cells model inside the microplate was performed by using a custom-built frequency domain optical coherence tomography (FD-OCT).

4.3.2 Synthesis of heavy-atom-free aza-BODIPY (AB-OH)

The synthetic aza-BODIPY (AB-OH) was synthesized according to **Figure 4.2**, and all synthesized compounds were characterized by ^1H and ^{13}C -NMR, and HRMS MALDI-TOF to confirm their structures.

4.3.2.1 Synthesis of (E)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (1)

4'-Methoxyacetophenone (5.00 g, 33.29 mmol), 4-hydroxy-3-methoxybenzaldehyde (5.06 g, 33.29 mmol), and KOH (9.34 g, 166.45 mmol) were dissolved in 80 mL methanol at room temperature. Then, the reaction mixture was stirred for 8 h. After completion, the reaction progress was monitored by TLC. After that, methanol was removed under reduced pressure. The crude product was purified by silica gel column chromatography using 30% ethyl acetate/hexane as solvent to obtain a yellow solid compound (5.89 g, 63%). ^1H NMR (500 MHz, CDCl_3) δ 8.12 (d, J = 9 Hz, 2H), 7.83 (d, J = 15 Hz, 1H), 7.48 (d, J = 15.5 Hz, 1H), 7.30 (d, J = 8 Hz, 1H), 7.22 (s, 1H), 7.07 (d, J = 8.5 Hz, 2H), 7.04 (d, J = 8 Hz, 1H), 6.05 (s, 1H), 4.05 (s, 3H), 3.98 (s, 3H) ppm. ^{13}C NMR (125 MHz, CDCl_3) δ 188.86, 163.29, 148.12, 146.80, 144.36, 131.34, 130.73, 127.68, 123.16, 119.57, 114.86, 113.80, 110.06, 56.03, 55.50 ppm; HRMS MALDI-

TOF (m/z): the calculated value (calcd) for $C_{17}H_{16}O_4Na$ ($[M+Na^+]$): 307.0946, found 307.0950.

4.3.2.2 Synthesis of 3-(4-hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)-4-nitrobutan-1-one (2)

Compound **1** (1.02 g, 4.11 mmol) and KOH (0.92 g, 16.43 mmol) were dissolved in methanol at room temperature. After that, nitromethane (1 ml, 16.43 mmol) was added to the reaction and stirred at reflux for 18 h. After cooling to room temperature, methanol was removed under reduced pressure. The residue was purified by column chromatography eluting with 30% ethyl acetate/hexane to obtain as yellow oil product (0.85 g, 61 %). 1H NMR (500 MHz, MeOD- d_4) δ 8.02 (d, J = 1.5 Hz, 1H), 8.01 (s, 1H), 7.07 (d, J = 1.5 Hz, 1H), 7.05 (s, 1H), 6.97 (s, 1H), 6.82 (d, J = 8 Hz, 1H), 6.79 (d, J = 8.5 Hz, 1H), 4.92 (s, 1H), 4.81 (dd, J = 12.5, 9 Hz, 1H), 4.14 (q, 1H), 3.94 (s, 3H), 3.91 (s, 3H), 3.53 (d, J = 7 Hz, 1H), 3.44 (d, J = 7 Hz, 1H) ppm. ^{13}C NMR (125 MHz, MeOD- d_4) δ 196.92, 163.94, 147.64, 145.52, 131.10, 130.20, 129.57, 119.83, 115.02, 113.50, 111.20, 79.68, 55.06, 54.68, 41.00, 39.64 ppm. HRMS MALDI-TOF (m/z): the calculated value (calcd) for $C_{17}H_{16}O_4Na$ ($[M+Na^+]$): 368.1110, found 368.1124.

4.3.2.3 Synthesis of (Z)-4-(2-((3-(4-hydroxy-3-methoxyphenyl)-5-(4-methoxyphenyl)-1H-pyrrol-2-yl)imino)-5-(4-methoxyphenyl)-2H-pyrrol-3-yl)-2-methoxyphenol (3)

Ammonium acetate (9.82 g, 12.74 mmol) was added into a solution of **2** (0.88 g, 2.54 mmol) butanol (50 mL) at room temperature and continued stirred at reflux for 18 h. After cooling to room temperature, the mixture was extracted with ethyl acetate (20 ml, 3 times). The combined organic phase was washed with water (50 ml) and brine solution (50 ml), dried over anhydrous $MgSO_4$, filtered, and evaporated. The crude product was purified by silica gel column chromatography using 30% ethyl acetate/hexane as solvent to obtain a green solid compound (0.67g, 89%). 1H NMR (500 MHz, DMSO- d_6) δ 9.38 (s, 2H), 8.09 (d, J = 7 Hz, 4H), 7.67 (d, J = 8 Hz, 2H), 7.62 (s, 2H), 7.53 (s, 2H), 7.28 (d, J = 7 Hz, 4H), 6.91 (d, J = 8 Hz, 2H), 3.97 (s, 6H), 3.80 (s, 6H) ppm. ^{13}C NMR (125 MHz, DMSO- d_6) δ 161.58, 154.15, 149.01, 148.02, 147.65, 142.17, 128.59, 125.64, 124.66, 122.54, 115.84, 115.45, 114.14, 113.29, 56.01,

55.99 ppm; HRMS MALDI-TOF (m/z): the calculated value (calcd) for $C_{36}H_{31}N_3O_6$ ($[M^+]$): 602.2246, found 602.2274.

4.3.2.4 Synthesis of 5,5-difluoro-1,9-bis(4-hydroxy-3-methoxyphenyl)-3,7-bis(4-methoxy phenyl)-5H-dipyrrolo[1,2-c:2',1'-f][1,3,5,2]triazaborinin-4-ium-5-uide (AB-OH)

Compound **3** (0.33 g, 0.55 mmol) was dissolved in the mixture of dry CH_2Cl_2 , *N,N*-Diisopropylethylamine, and DIPEA (0.96 mL, 5.48 mmol) and stirred for 30 min at room temperature under nitrogen atmosphere. $BF_3 \cdot OEt_2$ (0.68 mL, 5.48 mmol) was then added to the reaction mixture and carried out overnight. The final product **AB-OH** (0.23 g, 67%) as a blue solid was acquired by silica gel column chromatography eluted with 30% ethyl acetate/hexane. 1H NMR (500 MHz, $DMSO-d_6$) δ 9.76 (s, 2H), 8.20 (d, J = 9 Hz, 4H), 7.80 (d, J = 1.5 Hz, 1H), 7.78 (d, J = 1.5 Hz, 1H), 7.72 (s, 2H), 7.51 (s, 2H), 7.20 (d, J = 9 Hz, 4H), 6.97 (d, J = 8 Hz, 2H), 3.95 (s, 6H), 3.87 (s, 6H) ppm. ^{13}C NMR (125 MHz, $DMSO-d_6$) δ 162.06, 157.05, 149.36, 148.37, 144.75, 143.12, 131.95, 124.20, 124.06, 123.58, 118.30, 116.23, 114.74, 113.62, 56.20, 56.01 ppm; HRMS MALDI-TOF (m/z): the calculated value (calcd) for $C_{36}H_{30}BF_2N_3O_6$ ($[M^+]$): 649.2196, found 649.2317.

4.3.3 Preparation of AB-OH-based nanoparticles

The mixture of poly(ethylene glycol)-succinic acid polymer (mPEG-SA, 0.33 g, 0.07 mmol), EDC-HCl (0.04 g, 0.07 mmol) and **AB-OH** (0.04 g, 0.07 mmol) were dissolved in 2 mL of DMSO. The resulting solution was allowed to react overnight at room temperature. After that, 20 mL of DI was added to the crude product. Then, the aqueous solution of NPs was loaded into a dialysis bag and dialyzed against DI water for 24 h. After that, 1.40 mL of the aqueous solution was transferred into a 1.5 mL Eppendorf tube and centrifuged at 4 °C 13,000 rpm for 1 h. The resulting NP aqueous solution was further filtered through a 0.2 μm filter and then freeze-dried to give a green solid **AB-OH@mPEG-SA** (62%).

For the characterization of prepared NPs, the particle size was determined by dynamic light scattering (DLS) (Malvern Model Zetasizer-ZS). The morphology of obtained NPs was investigated by JEM-2100-Plus transmission electron

microscope (TEM; JEOL). The concentration of the NPs was calculated from UV–Vis–NIR absorbance at 720 nm based on a calibration curve of free dye (Shimadzu/UV-1900i).

To test the colloidal stability, NPs were dissolved in various mediums including 0.1 M PBS pH 7.4, 0.1 M PBS pH 5.0, DI water, RPMI, and FBS. In addition, the NPs' size and zeta potential were also measured at different time points (0, 1, 3, 5, 7, 14, and 30 days) in PBS pH 7.4.

4.3.4 Photophysical properties

The stock solutions of **AB-OH** and **AB-OH@mPEG-SA** NPs were prepared in DMSO. The stock solution of **AB-OH** and NPs were added to 3.0 mL of organic solvents in a 3.5 mL quartz cuvette (final concentration $\sim 5 \mu\text{M}$). All the UV-vis-NIR absorption and fluorescence spectra were recorded on Cary Series UV-Vis-NIR spectrophotometer and PTI QuantaMaster 500 – Near Infra-Red Photoluminescence System (HORIBA Scientific), respectively. For fluorescence experiments, the emission spectra were recorded at an excitation wavelength of 650 nm, excitation slit widths = 5 nm, and emission slit = 5 nm.

The fluorescence quantum yields (Φ_f), defined as the ratio of the number of photons emitted to the number of photons absorbed, were calculated using eqn (1) and related to Zinc phthalocyanine (ZnPc) in pyridine as a standard (std) ($\Phi_{\text{std}} = 0.30$).

$$\Phi_f = \Phi_{\text{std}} (A_{\text{sample}}/A_{\text{std}})(I_{\text{std}}/I_{\text{sample}})(\eta_{\text{sample}}/\eta_{\text{std}})^2$$

where F denotes the fluorescence quantum yield, A is the peak area of emission, I is the absorbance at the excitation wavelength, and η is the solvent reflective index.

4.3.5 Singlet oxygen generation

Indirect measurement of singlet oxygen generation was evaluated using 1,3-diphenylisobenzofuran (DPBF), a singlet oxygen scavenger. Singlet oxygen generation was determined by irradiation using a 660 nm red LED lamp (8.7 mW/cm^2). The solutions of **AB-OH@mPEG-SA** NPs were prepared in THF at a concentration of 2

μM . 1,3-Diphenylisobenzofuran (DPBF, 1 mM) was prepared in DMSO and diluted to 80 μM in THF. Then, the solutions were irradiated by a 660 nm red LED lamp at a power density of 8.7 mW cm^{-2} every 10 s for 100 s. The decreasing absorbance at 415 nm was measured with a UV-Vis-NIR spectrophotometer (Shimadzu/UV-1900i).

4.3.6 Cell culture

Murine breast cancer (4T1) cells and human embryonic kidney 293 (HEK-293) cells were cultured on 75 cm^2 culture flasks in culture medium; Roswell Park Memorial Institute (RPMI-1640, HYCLONE) for 4T1, Dulbecco's Modified Eagle Medium/High glucose (DMEM/HIGH GLUCOSE, GE Healthcare Life Sciences HyClone Laboratories) for HEK-293. containing 10% fetal bovine serum (FBS, Gibco) and 1% Penicillin Streptomycin Solution 100X (CORNING). All the cells were cultured at 37 °C in a humidified 95% air, 5% CO_2 atmosphere.

4.3.7 Live cell imaging

4T1 cells were seeded in a 8-well chambered coverglass (Nunc Lab-Tek II Chamber Slide) with non-removable wells at 7×10^3 cells per well and incubated for 24 h at 37 °C under 5% CO_2 . After that, cell culture media were removed and the cells were treated with 2 μM of **AB-OH** and **AB-OH@mPEG-SA** for 0, 1, 3, 6, and 24 h. After incubation, the cells were washed with 0.01 M PBS (3 times) and treated with 0.5 mg mL^{-1} of Hoechst 33342 (DNA fluorescent staining, Thermo Fisher Scientific) containing media for 10 min. The cells were imaged by a Laser Scanning Confocal Microscope (LSCM, Nikon A1Rsi) with a 60X oil immersion objective lens. **Free AB-OH** and **AB-OH@mPEG-SA** were excited by a 643 nm laser (Em: 663-738) and Hoechst 33342 was excited by a 408 nm laser (Em: 425-475). All data were analyzed by ImageJ and presented quantitative fluorescent intensity as corrected total cell fluorescence (CTCF), mean $\pm$ SD (n = 30, 30 cells/set).

For the colocalization experiment, seeded cells in a 8-well chambered coverglass were incubated with 2 μM of **AB-OH@mPEG-SA** in a complete RPMI medium for 24 h, washed three times with PBS and incubated with media containing 1 μM of organelles trackers; LysoTracker™ Green DND-26 (Thermo Fisher Scientific), MitoTracker™ Green FM (Thermo Fisher Scientific), ERTracker™ Green (BODIPY™ FL Glibenclamide, Thermo Fisher Scientific), and C6-NBD Ceramide (Golgi tracker, Avanti

Polar Lipids) for 20 min. The cells were washed again before staining with Hoechst 33342 for 10 min. After that, cells were visualized under LSCM: **AB-OH@mPEG-SA** was excited by a 643 nm laser (Em: 663-738), organelles trackers were excited by a 488 nm laser (Em: 500-550 nm), and Hoechst 33342 was excited by a 408 nm laser (Em: 425-475). To quantify the colocalization of Pearson's correlation coefficient for **AB-OH@mPEG-SA** with organelles trackers, we obtained the intensities of the purple and green channels from each pixel of the confocal images and calculated Pearson's correlation coefficient using ImageJ.

4.3.8 Light-induced cytotoxicity

The cytotoxicity of nanoparticles toward 4T1 cells was assessed through the MTT assay. The 4T1 cells were seeded into 96-well culture plates at the cell density to 1×10^4 per well and incubated at 37 °C in a 5% CO₂ atmosphere for 24 h. Thereafter, the cells were treated with **AB-OH** and **AB-OH@mPEG-SA** at concentrations of 0, 0.15, 0.3, 0.6, 1.25, 2.5, 5, and 10 μ M in a complete culture medium. After another 24 h of incubation, the cells were washed three times with 0.01 M PBS, and fresh complete cell culture media were added before being irradiated by a 660 nm red LED lamp (60 mW cm⁻²) for various time points (0, 15, and 30 min), followed by an additional 24-h incubation. Then, the cells were treated with methylthiazolyldiphenyl-tetrazolium bromide (MTT reagent, 100 μ L, 0.5 mg mL⁻¹, Sigma-Aldrich) for 2-3 h. After media removal, dimethyl sulfoxide (DMSO) was added to dissolve the formazan product. The cell's viabilities were detected through UV-vis absorption of formazan at wavelength 560 nm using a microplate reader (BMG Labtech/SPECTROstar Nano), which % viability was calculated from $100 \times (A_{\text{sample}}/A_{\text{control}})$; where A = absorbance at 560 nm.

4.3.9 Live/dead staining assay

4T1 cells were seeded into 6-well culture plates at a density of 2×10^5 cells/well and incubated for 24 h at 37 °C under 5% CO₂. After that, all the cells were treated with **AB-OH** and **AB-OH@mPEG-SA** (5 μ M) for 24 h. After washing with PBS, the cells were irradiated by a 660 nm red LED lamp (60 mW cm⁻²) for 30 min and re-incubated for another 24 h. Then, the treated cells were stained with calcein

acetoxymethyl (Calcein-AM) and propidium iodide (PI) for 20 min. Non-irradiated cells were used as a control. The cells were visualized under fluorescent microscopy. Calcein-AM (490 nm excitation and 515 nm emission filters) showed live cells in green color, and PI (535 nm excitation and 615 nm emission filters) showed dead cells in red color.

4.3.10 Intracellular ROS and singlet oxygen generation

To examine intracellular singlet oxygen production, 4T1 cells were seeded on an 8-well chambered coverglass (1×10^4 cells/well) and incubated at 37 °C under 5% CO₂ for 24 h. Cultured media were replaced with free **AB-OH** and **AB-OH@mPEG-SA** NPs for 24 h. Subsequently, the cells were washed with PBS (3 times) and incubated with either singlet oxygen sensor green (SOSG, 20 mM, Thermo Fisher Scientific) or 2,7-dichloro-dihydro-fluorescein diacetate (DCFH-DA, 20 mM, Sigma-Aldrich) for 2 h. After washing with PBS, the cells were irradiated by a 660 nm red LED lamp at a power density of 60 mW cm⁻² for 10 min and re-incubated for 20 min. Non-irradiated cells served as a control. Before imaging, the cells were washed with PBS (3 times) followed by nucleus staining with 1.0 μM of Hoechst 33342. Imaging was performed using LSCM equipped with a 60X oil immersion objective lens (Nikon A1Rsi). The fluorescence signals of products of DCFH-DA, 2,7-dichloro-dihydro-fluorescein (DCF), and SOSG were detected using a 488 nm excitation laser (emission: 500–550 nm), while Hoechst 33342 fluorescence was visualized with a 408 nm excitation laser (emission: 425–475 nm).

4.3.11 Apoptosis detection by flow cytometry

4T1 cells were seeded into a 6-well plate at a density of approximately 2×10^5 cells/well for 24 h at 37 °C under 5% CO₂. The cells were treated with free dye **AB-OH** and **AB-OH@mPEG-SA** NPs in a complete medium for 24 h. Following treatment, the cells were washed with 0.01 M PBS 3 times. Subsequently, the cells were irradiated with a red 660 nm LED lamp at a power density of 60 mW cm⁻² for 10 min and re-incubated for another 24 h. Thereafter, the cells were trypsinized and washed three times with ice-cold 0.01 M PBS buffer (pH 7.4) by centrifugation at 4000

rpm at 4 °C for 3 min. The cell pellets were then resuspended in 0.2 mL of 1X Annexin binding buffer (Thermo Fisher Scientific from Tali™ Apoptosis Kit). Then, 10 µL of Annexin V fluorescein conjugate (Annexin V Alexa Flour™ 488, Thermo Fisher Scientific from Tali™ Apoptosis Kit) was added to the cell suspension, followed by a 15-min incubation at room temperature. Subsequently, 2 µL of 1 mg/mL Propidium iodide (PI, Sigma-Aldrich) was added to the cell samples on ice. The cells were kept on ice during the flow cytometry process. Then, 1×10^4 events were analyzed by flow cytometry using an Attune NxT Flow Cytometer (Thermo Fisher Scientific).

4.3.12 3D-cell culture

The *in vitro* studies on three-dimensional (3D) breast cancer tumor models were also tested using the same procedure described in Chapter III and similar conditions in 2D-cell culture as mentioned above.

4.4 Results and discussion

4.4.1 Design, synthesis and characterization of AB-OH

We designed and synthesized the novel aza-BODIPY (**AB-OH**) based on electron-donating and -accepting (D-A) system to create low-bandgap organic dyes with red-shifted absorption for deep penetration *in vivo* (Hendriks, Li, Wienk, and Janssen, 2014). Herein, the methoxy and hydroxyl moieties acted as the electron donor and the aza-BODIPY core acted as the electron acceptor. **AB-OH** was synthesized according to classical aza-BODIPY synthetic methods as shown in **Figure 4.2** (Bodio, Denat, and Goze, 2019; Ge and O'Shea, 2016; Shi et al., 2020). In the procedure, the chalcone derivative **1** can be simply prepared via an aldol condensation reaction between 4-hydroxy-3-methoxybenzaldehyde and 4'-methoxyacetophenone. After that, the resulting chalcone underwent Michael addition of nitromethane to provide **Y**-nitro-substituted ketone product (**2**), which was then reacted with an excess amount of ammonium acetate to give aza-dipyrrromethene (**3**). Finally, the complexation of **3** with boron trifluoride etherate (BF₃·OEt₂) yields **AB-OH** as a final product. The intermediates and final compound were fully confirmed by ¹H and ¹³C NMR spectroscopic techniques as shown in the NMR spectra in the supporting information (SI). In addition, the detection of the

molecular ions by positive-mode electrospray mass spectrometry at $m/z = 649.2317$ was used to further confirm the identity of **AB-OH** as indicated in supporting information.

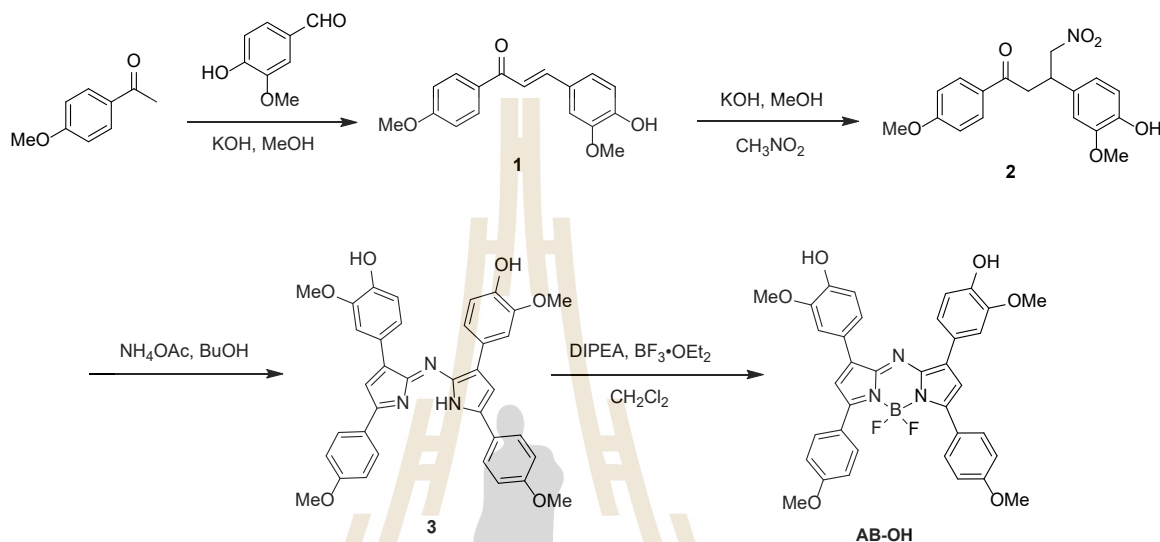


Figure 4.2 Synthetic procedure of free dye **AB-OH**.

4.4.2 Photophysical properties of **AB-OH**

We first investigated the photophysical properties of **AB-OH** using a UV-Vis-NIR and fluorescence spectrophotometer in different solvents as indicated in **Table 4.1** and **Figures 4.3A, B**. **AB-OH** features absorption maxima varying from 693 to 697 nm depending on the polarity and proticity of solvents with a high molar extinction coefficient. Interestingly, the dye exhibited strong fluorescent signals in toluene and dichloromethane (DCM), which are low polar solvents, with emission maxima at the center around 720 nm. In addition, **AB-OH** displayed higher fluorescence quantum yields (Φ_f) in low polarity solvents (toluene and DCM). Owing to efficient ICT from donor units to acceptor units, **AB-OH** showed weak fluorescence with low fluorescence quantum yield in comparison to Zn-phthalocyanine (Zn-Pc) as a reference ($\Phi_f = 0.30$ in pyridine) in high polar solvents, including acetone, acetonitrile, and methanol.

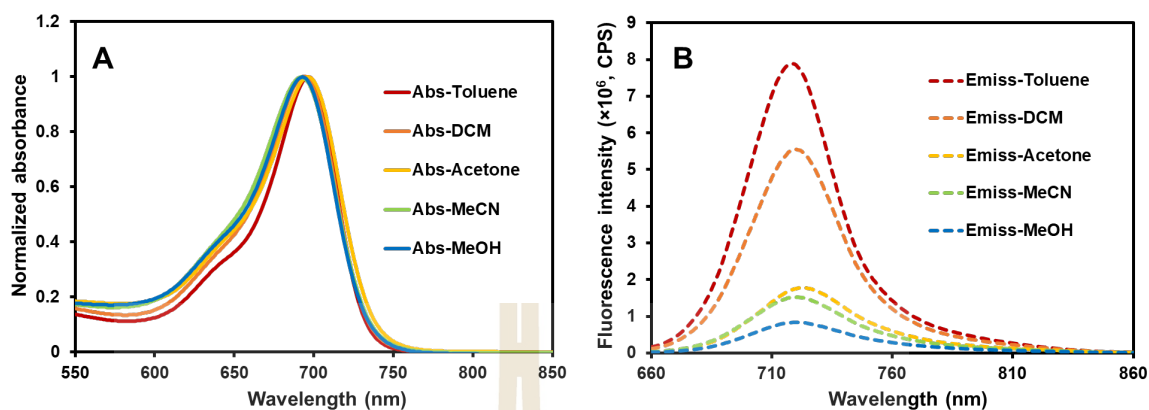


Figure 4.3 Photophysical properties of AB-OH. (A) absorption and (B) emission spectra in various solvents (excitation wavelength = 650 nm).

Table 4.1 Photophysical properties of free AB-OH (5 μ M).

Solvents ^a	λ_{abs}^b (nm)	ϵ ($\text{M}^{-1} \text{cm}^{-1}$)	λ_{em}^c (nm)	$\Delta\lambda^d$ (nm)	Φ_f^e
Toluene	700	3.0×10^4	719	19	0.145
DCM	695	3.1×10^4	720	25	0.104
Acetone	697	3.2×10^4	722	25	0.031
MeCN	693	2.9×10^4	719	26	0.025
MeOH	693	3.1×10^4	719	26	0.014

^a Solvents were arranged according to increasing polarity from top to bottom; DCM = Dichloromethane, MeCN = Acetonitrile, MeOH = Methanol. ^b λ_{abs} = absorption maximum wavelength. ^c λ_{em} = emission maximum wavelength (Excitation wavelength = 650 nm). ^d $\Delta\lambda$ = stokes shifts ($\lambda_{\text{em}} - \lambda_{\text{abs}}$). ^e Φ_f = fluorescence quantum yields estimated by using Zn-phthalocyanine in pyridine ($\Phi_f = 0.30$).



To improve the biocompatibility and tumor-targeting ability of **AB-OH** to realize biological applications, the nanoparticles (NPs) were prepared by conjugation of **AB-OH** with mPEG-SA5000 in a 1:1 ratio using EDC as a coupling reagent (**Figure 4.4**). After conjugation, the resulting NP was well dispersed in water, as demonstrated in the green solution which was different from the free dye (**Figure 4.5E**). Additionally, Fourier Transform Infrared Spectroscopy (FTIR) analysis of free **AB-OH**, mPEG-SA, and **AB-OH@mPEG-SA** NPs is demonstrated in **Figure 4.5F**, verifying the formation of nanoparticles. For FTIR spectra, aromatic hydrocarbons show absorptions in the regions $1600\text{--}1585\text{ cm}^{-1}$ and $1500\text{--}1400\text{ cm}^{-1}$ due to carbon-carbon stretching vibrations in the aromatic ring. Phenols also show a characteristic broad IR absorption at 3500 cm^{-1} due to the --OH group, as well as the usual 1500 and 1600 cm^{-1} aromatic band. Finally, the concentration of the dye in NP was calculated from the UV-Vis-NIR absorbance based on an **AB-OH** calibration curve as shown in the supporting information.

4.4.3 Synthesis and characterization of AB-OH@mPEG-SA

To improve the biocompatibility and tumor-targeting ability of **AB-OH** to realize biological applications, the nanoparticles (NPs) were prepared by conjugation of **AB-OH** with mPEG-SA5000 in a 1:1 ratio using EDC as a coupling reagent (**Figure 4.4**). After conjugation, the resulting NP was well dispersed in water, as demonstrated in the green solution which was different from the free dye (**Figure 4.5E**). Additionally, Fourier Transform Infrared Spectroscopy (FTIR) analysis of free **AB-OH**, mPEG-SA, and **AB-OH@mPEG-SA** NPs is demonstrated in **Figure 4.5F**, verifying the formation of nanoparticles. For FTIR spectra, aromatic hydrocarbons show absorptions in the regions $1600\text{--}1585\text{ cm}^{-1}$ and $1500\text{--}1400\text{ cm}^{-1}$ due to carbon-carbon stretching vibrations in the aromatic ring. Phenols also show a characteristic broad IR absorption at 3500 cm^{-1} due to the -OH group, as well as the usual 1500 and 1600 cm^{-1} aromatic band. Finally, the concentration of the dye in NP was calculated from the UV-Vis-NIR absorbance based on an **AB-OH** calibration curve as shown in the supporting information.

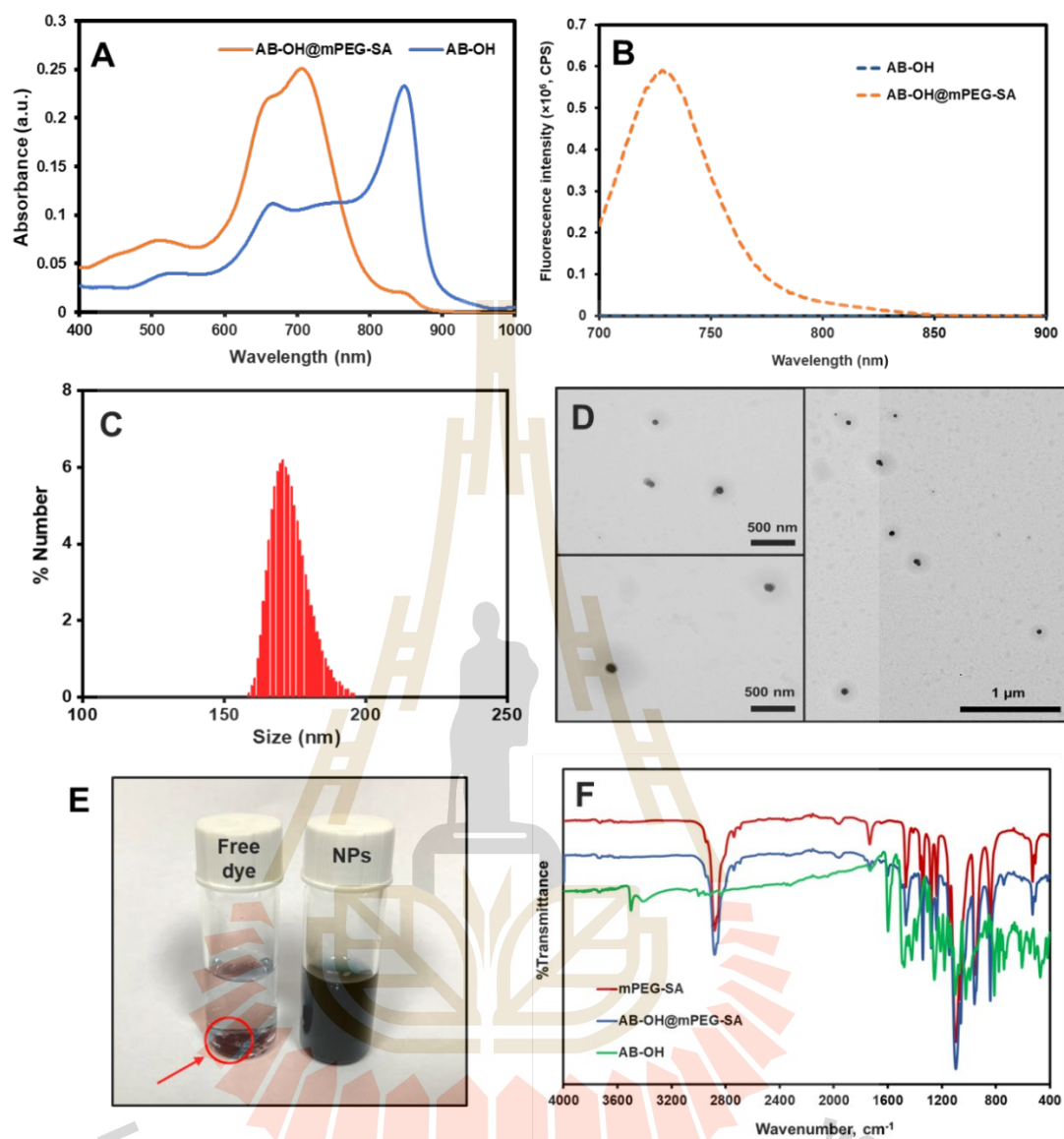


Figure 4.5 Photophysical properties and characterization of AB-OH@mPEG-SA NPs. (A) Normalized absorbance and (B) emission spectra in DI water (Excitation wavelength = 650 nm). (C) Hydrodynamic size distribution histogram in DI water measured by DLS. (D) TEM image. (E) vials containing (i) free dye AB-OH and (ii) AB-OH@mPEG-SA NPs suspended in DI water. (F) FTIR of mPEG-SA, AB-OH@mPEG-SA NPs, and free dye AB-OH.

The absorbance and fluorescence spectra of NPs were measured in DI water compared to free dye. As demonstrated in **Figures 4.5A and B**, the NPs absorb and emit in the near-infrared range, with an absorption maximum of 706 nm and an emission maximum of 730 nm. The resulting nanoparticles demonstrated broad absorption and emission spectra compared to those of the free **AB-OH** dye in organic solvents (**Figure 4.3**). Their spectra differed from those of the free dye **AB-OH** in water, which exhibited flattened absorption spectra and no detectable fluorescence signal in the emission spectra (**Figure 4.5B**). This indicates that aggregation occurred in water, making it unsuitable for biological applications. These results indicated that the nanomaterials could improve the photophysical properties of **AB-OH** in an aqueous solution. As shown in **Figures 4.5C and 4.5D**, the nanoparticles had a uniform particle size and good spherical morphology, which enabled the passive targeting of **AB-OH@mPEG-SA** NPs into tumor sites via the EPR effect (Kang et al., 2020; Maeda, Bharate, & Daruwalla, 2009). Moreover, the TEM image revealed a dispersed and isolated, spherical-shaped morphology of the obtained NPs (**Figure 4.5D**).

Table 4.2 Characteristics of **AB-OH@mPEG-SA** NPs in DI water, PBS at pH 5.0, PBS at pH 7.4, RPMI and FBS by DLS and zeta potential measurements.

Entry	Medium	DLS size (nm) (n = 3)	PDI (n = 3)	Zeta potential (mV) (n = 3)
1	DI water	192.0 ± 0.8	0.292 ± 0.025	-8.24 ± 1.03
2	PBS at pH 5.0	188.6 ± 11.5	0.377 ± 0.023	-6.38 ± 1.17
3	PBS at pH 7.4	164.9 ± 4.6	0.282 ± 0.024	-16.07 ± 1.67
4	RPMI	206.8 ± 6.9	0.456 ± 0.037	-5.04 ± 0.15
5	FBS	165.2 ± 1.3	0.859 ± 0.005	-9.05 ± 0.21

Next, we further evaluated whether the NPs had colloidal stabilities in biological environments, which is critical for the further clinical translation of **AB-OH@mPEG-SA** nanomaterials. We selected five different aqueous solutions to study the colloidal stabilities of our NPs, including DI water, phosphate buffer saline (PBS) pH 5 and pH 7.4, Roswell Park Memorial Institute (RPMI) 100/640 culture medium, and fetal bovine serum (FBS). As shown in **Table 4.2**, the size analysis using DLS displayed the hydrodynamic (HD) of **AB-OH@mPEG-SA** NPs average sizes ranging from 165 to 200 nm with an excellent polydispersity index (PDI) and a negative surface charge in various mediums, which was favorable for the stability of nanoparticles due to the electrostatic repulsion. Moreover, the colloidal stability of the NPs was also investigated in 0.1 M PBS (pH 7.4) for a longer time incubation (**Table 4.3**), the DLS sizes and zeta-potentials of the NPs were slightly changed in the medium up to 30-day incubation, demonstrating high colloidal stability of **AB-OH@mPEG-SA** NPs in aqueous solutions. Furthermore, when the NPs in PBS at pH 7.4 were irradiated with a 30-minute 660 nm red light lamp, their size changed negligibly compared to the non-irradiated NP solutions, indicating that the NPs have high stability even after prolonged irradiation.

Table 4.3 Colloidal stability of **AB-OH@mPEG-SA** NPs in 0.1 M PBS (pH 7.4) at different time points by DLS and zeta potential measurements.

Day	DLS size (nm) (n = 3)	PDI (n = 3)	Zeta potential (mV) (n = 3)
0	164.9 ± 4.6	0.282 ± 0.024	-16.07 ± 1.67
3	209.3 ± 1.4	0.277 ± 0.008	-3.28 ± 0.82
7	183.8 ± 17.8	0.310 ± 0.021	-3.16 ± 0.78
14	181.4 ± 2.5	0.164 ± 0.003	-1.59 ± 0.42
30	219.0 ± 1.5	0.307 ± 0.013	-1.16 ± 0.45

4.4.4 Photodynamic properties of AB-OH-based nanoparticles

The singlet oxygens ($^1\text{O}_2$) are cytotoxic and can effectively kill the cancer cells. Prior to the cell experiment, the $^1\text{O}_2$ production efficiencies of **AB-OH** and **AB-OH@mPEG-SA** NPs were first performed in solution using 1,3-diphenylisobenzofuran (DPBF, singlet oxygen indicator) as a probe. When DPBF reacts with $^1\text{O}_2$, it will change its spectroscopic properties due to the loss of the extended π -electron system as indicated by the decreasing DPBF absorbance at 415 nm. The nanoparticles or free dye were mixed with DPBF under a 660 nm LED lamp irradiation (60 mW cm^{-1}) for 100 seconds. As shown in **Figures 4.6C** and **4.6D**, the absorption of DPBF decreased dramatically with the extension of light irradiation in the presence of **AB-OH@mPEG-SA** NPs, whereas **AB-OH** alone resulted in a slight change under the same conditions (**Figures 4.3B**). In addition, DPBF displayed a negligible change when compared to free dye or NPs, as displayed in **Figure 4.6A**. These results indicated that nanostructure could improve the efficiency of singlet oxygen generation.

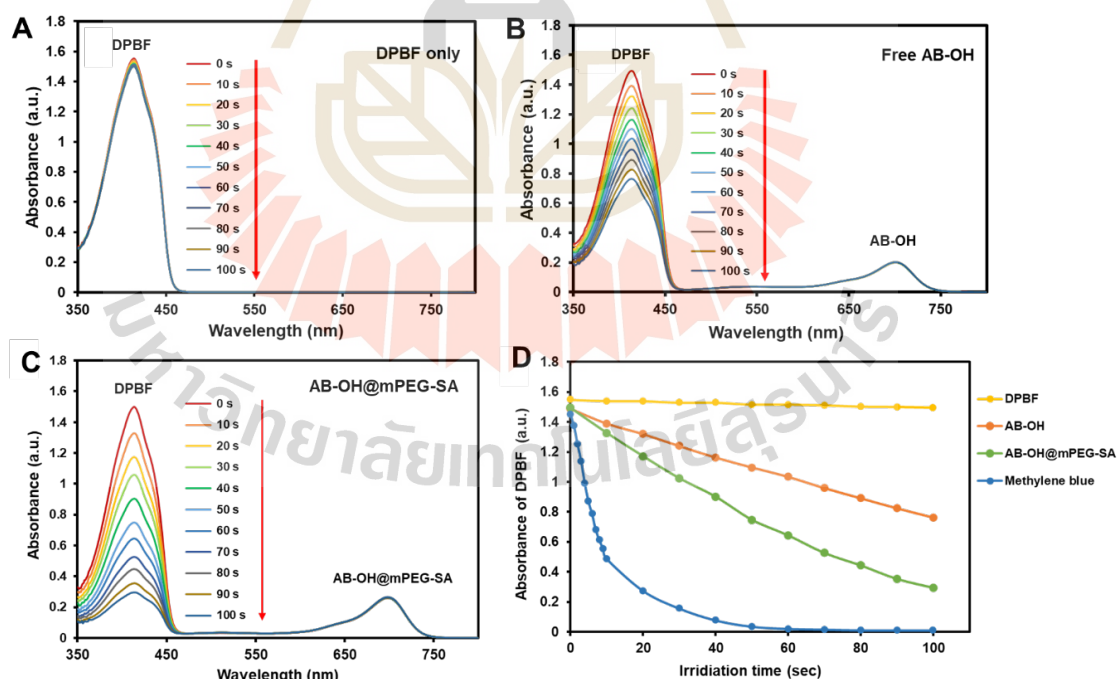


Figure 4.6 Reduction in UV-Vis-NIR absorbance of the DPBF (60 μM) solution with (A) DPBF only, (B) Free **AB-OH**, and (C) **AB-OH@mPEG-SA** containing 2 μM **AB-OH** under light irradiation for 100 s in THF. (D) time-dependent UV-Vis-NIR absorbance change of

DPBF with **AB-OH@mPEG-SA** NPs containing 2 μM **AB-OH** compared to free **AB-OH**, methylene blue, and DPBF in THF under light irradiation for 100 s.

Furthermore, the photothermal effect of **AB-OH@mPEG-SA** NPs was also investigated. The nanomaterials with different NP contents (0, 50, 250, 500, 750, and 1,000 $\mu\text{g mL}^{-1}$) were illuminated with a 660 nm LED lamp for 10 min. As depicted in the supporting information, the temperature promotion of **AB-OH@mPEG-SA** NP solution illustrated concentration-dependent behavior. At the concentration of 500 $\mu\text{g mL}^{-1}$, the temperature of the NP solution increased by 12 $^{\circ}\text{C}$, whereas the temperature of DI water displayed a little heat under the same irradiation, indicating that **AB-OH@mPEG-SA** NP could be used as a mild photothermal agent in photothermal therapy (PTT).

4.4.5 Cell internalization of **AB-OH@mPEG-SA**

To investigate the effectiveness of the **AB-OH@mPEG-SA** NPs in cancer cell applications, the cellular uptake of resulting nanoparticles is critical for efficient cancer phototherapy *in vitro* and *in vivo*. We first carried out an intracellular internalization experiment to investigate the accumulation of free **AB-OH** and **AB-OH@mPEG-SA** NPs at various time points (0, 1, 3, 6, and 24 h) within 4T1 (murine breast cancer cells). As visualized under a laser scanning confocal microscope in **Figure 4.7**, confocal images of **AB-OH@mPEG-SA** NPs internalization within 4T1 cells revealed that the cellular uptake of NPs extended within the first 6 h of incubation. In addition, the NPs showed significantly enhanced cellular accumulation in 4T1 cells in comparison to free dye, implying that the nanostructure could be effectively accumulated in tumor cells. The results revealed that **AB-OH@mPEG-SA** NPs demonstrated good biocompatibility.

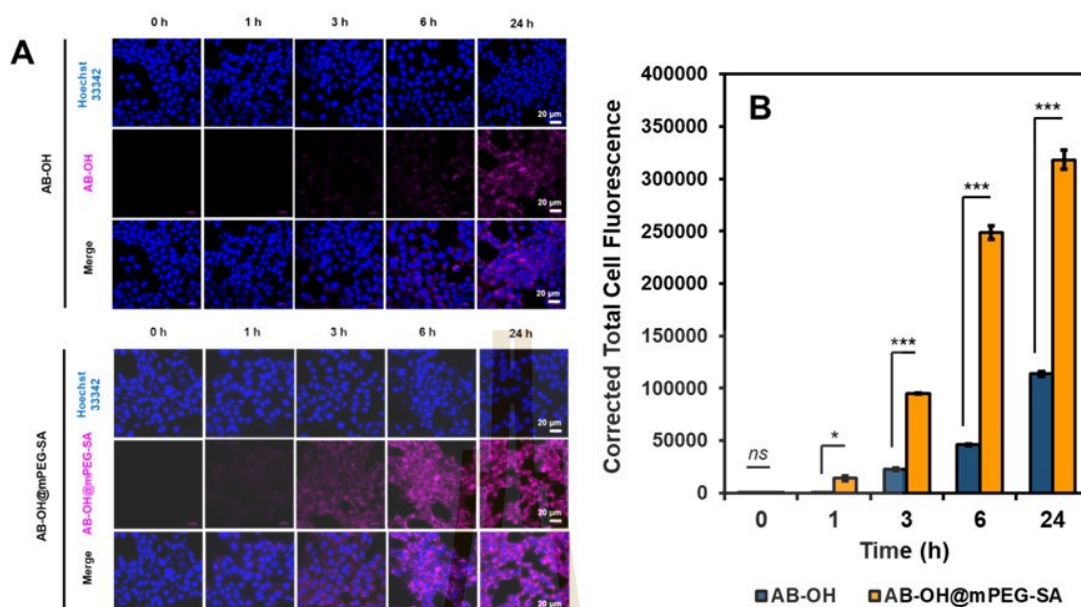


Figure 4.7 Live cell imaging. (A) Confocal and fluorescence microscopy images of 4T1 cells incubated with **AB-OH** (2 μ M) and **AB-OH@mPEG-SA** (containing 2 μ M **AB-OH**) depend on incubation times (0, 1, 3, 6, and 24 h) and with Hoechst 33342 (DNA fluorescent staining, blue). Ex: 408 nm for Hoechst 33342 and 643 nm for **AB-OH**; Em: 425–475 nm for Hoechst 33342 and 600–650 nm for **AB-OH**. Scale bars = 20 μ m. (B) Quantitative fluorescent intensity is represented as corrected total cell fluorescence (CTCF), which was quantified using ImageJ and represents the mean $\pm$ SD ($n = 3$, 30 cells/set). Statistical analysis is based on a T-test (*ns* = no significance, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Moreover, colocalization experiments were conducted in 4T1 cells to identify preferred organelles for **AB-OH@mPEG-SA** accumulation within the cells (Figure 4.8). **AB-OH@mPEG-SA** was managed within 4T1 and colocalized to varying degrees with LysoTracker green (Pearson's R-value = 0.740), MitoTracker Green (Pearson's R-value = 0.877), C6-NBD ceramide (Golgi tracker, Pearson's R-value = 0.469), and ERTracker Green (Pearson's R-value = 0.612). These results indicated that the probe was accumulated primarily in mitochondria and lysosomes, but not in the Golgi apparatus and endoplasmic reticulum.

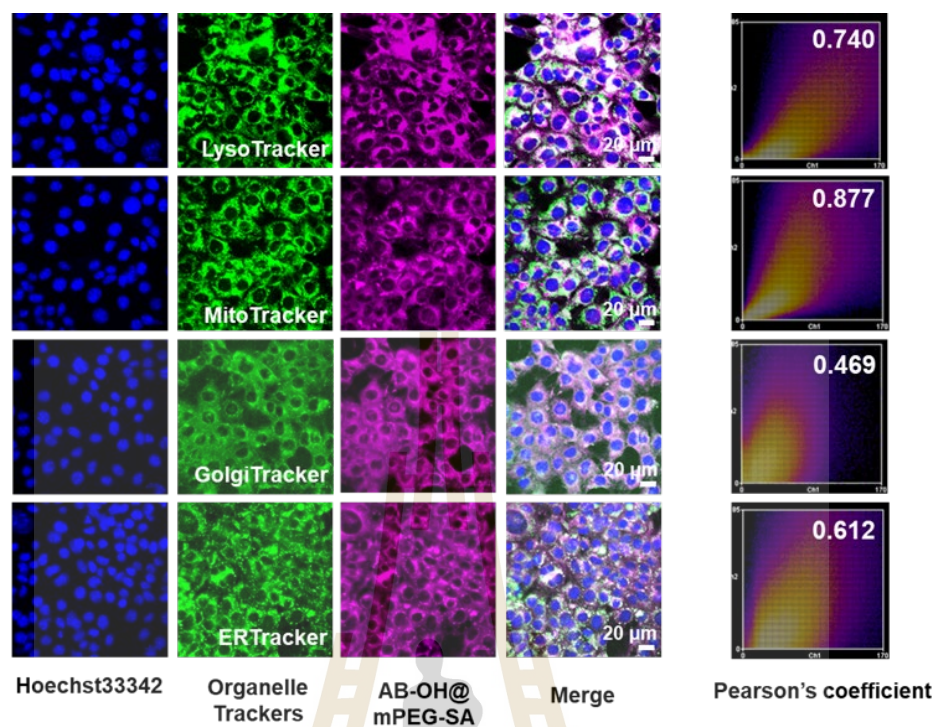


Figure 4.8 Colocalization staining of 4T1 treated with AB-OH@mPEG-SA NPs (2 μ M) at 24 h incubation with Hoechst 33342 and organelle tracker: LysoTracker green DND-26, MitoTracker Green FM, C6-NBD Ceramide (Golgi tracker, green), and ERTracker green. Ex: 408 nm for Hoechst 33342, 488 nm for organelle trackers, and 643 nm for AB-OH@mPEG-SA; Em: 425–475 nm for Hoechst 33342, 500–550 nm for organelle trackers, and 600–650 nm for AB-OH@mPEG-SA. Scale bars = 20 μ m.

4.4.6 *In vitro* phototherapeutic properties of AB-OH@mPEG-SA

The PDT efficiency of the nanoparticles was investigated under light and dark conditions against 4T1 cells by the MTT assay (Van Meerloo, Kaspers, and Cloos, 2011). As shown in **Figure 4.9A**, the cells that were exposed to AB-OH@mPEG-SA NPs under the irradiation of a 660 nm red LED lamp for 15 min dropped dramatically in a dose-dependent manner. The cell viability decreased to approximately 9% at a concentration of AB-OH@mPEG-SA NPs containing 5 μ M AB-OH. Additionally, the half-maximal inhibitory concentration (IC_{50}) of the NPs (IC_{50} = 0.13 μ M) under 15 min light irradiation demonstrated better phototoxicity than AB-OH alone (IC_{50} = 0.38 μ M). Furthermore, when the light dose was extended to 30 min

irradiation, the cell viability reduced gradually (**Figure 4.9B**). Moreover, the nanomaterials illustrated lower viabilities than free dye when compared at the same concentration in the presence of light illumination because the NPs can accumulate into the cancerous cells more than free dye, which agrees well with the cellular uptake experiment. While the cell survival remained at full viability without light irradiation. This suggests that the probe and its nanoparticles are non-toxic to both non-cancerous and cancerous cells under dark conditions.

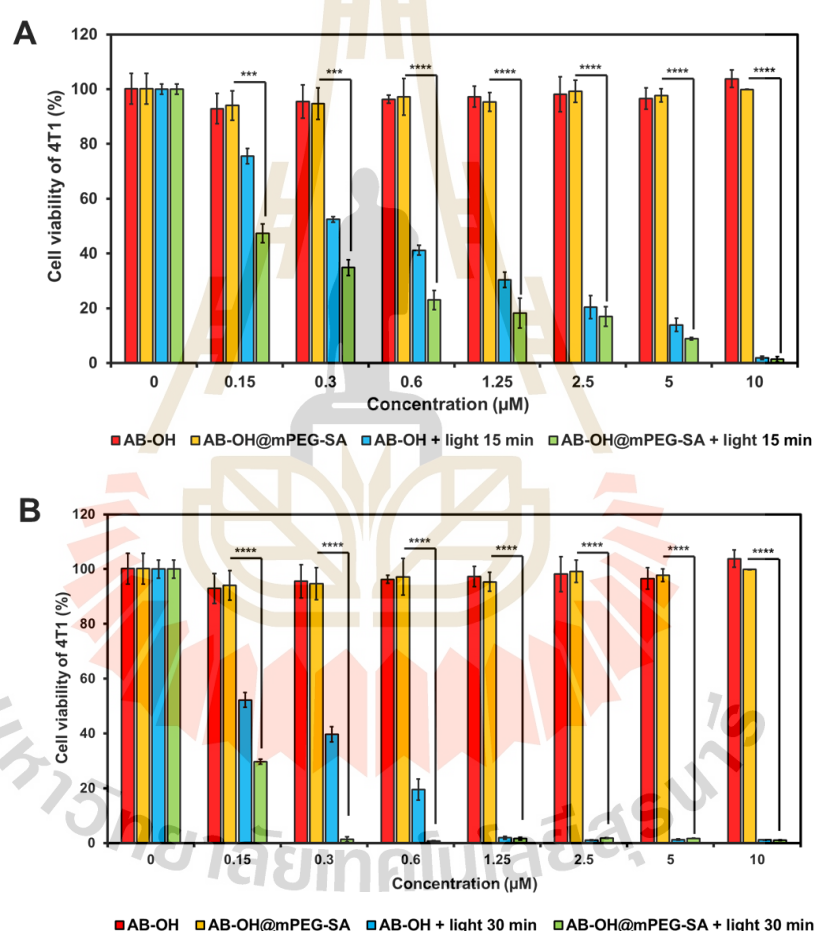


Figure 4.9 The relative cell viability of 4T1 cells with various concentrations of AB-OH and AB-OH@mPEG-SA NPs treatment under (A) 15 min and (B) 30 min light irradiation and dark conditions. Statistical analysis is based on the Student's t-test (*** $P < 0.001$, **** $P < 0.0001$) as compared to the cells treated with AB-OH@mPEG-SA NPs under light and dark conditions.

Additionally, the cytotoxicity of free **AB-OH** dye and **AB-OH@mPEG-SA** nanoparticles were also assessed in a normal cell line (HEK-293, human embryonic kidney cells). The cells were incubated with the materials for up to 24 h in the absence of light irradiation. The results indicated that the cells maintained high viability (> 90%) even at high concentrations as demonstrated in supporting information, implying that the probe and its nanoparticles are non-toxic to normal and cancer cells in dark conditions.

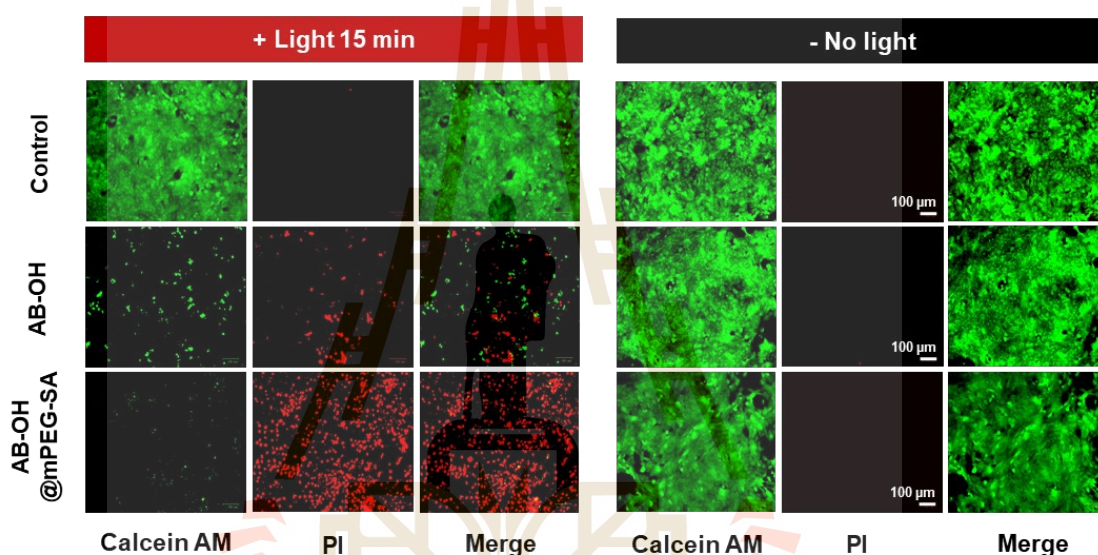


Figure 4.10 Live/dead viability/cytotoxicity co-staining images of **AB-OH** (2.5 μM) and **AB-OH@mPEG-SA** NPs (2.5 μM) followed by a 660 nm light irradiation for 15 min. The live cells were stained with green fluorescence, while dead cells were labeled with red fluorescence. Ex: 488 nm for Calcein AM and 535 nm for PI; Em: 500–550 nm for Calcein AM and 570–620 nm for PI. Scale bar = 100 μm .

Furthermore, the intracellular phototoxicity of the probes was further validated with live/dead viability/cytotoxicity experiments. The cells were treated with either **AB-OH** alone or **AB-OH@mPEG-SA** NPs before being exposed to 660 nm light for 30 min. Subsequently, the cells underwent staining with Calcein AM and propidium iodide (PI) to discriminate between viable and dead cells, respectively. As indicated in **Figure 4.10**, a significant proportion of the cells treated with the NPs

under light illumination showed predominantly red fluorescence, suggesting that most of the cells were dead. In contrast, all the control groups (untreated cells, light irradiation only, **AB-OH** only, and **AB-OH@mPEG-SA** NPs only) without light irradiation exhibited strong green fluorescence, demonstrating the high survival rate of cancer cells upon these treatments, which is consistent well with the findings observed in the cytotoxicity experiment, indicating an excellent phototherapeutic effect of **AB-OH@mPEG-SA**.

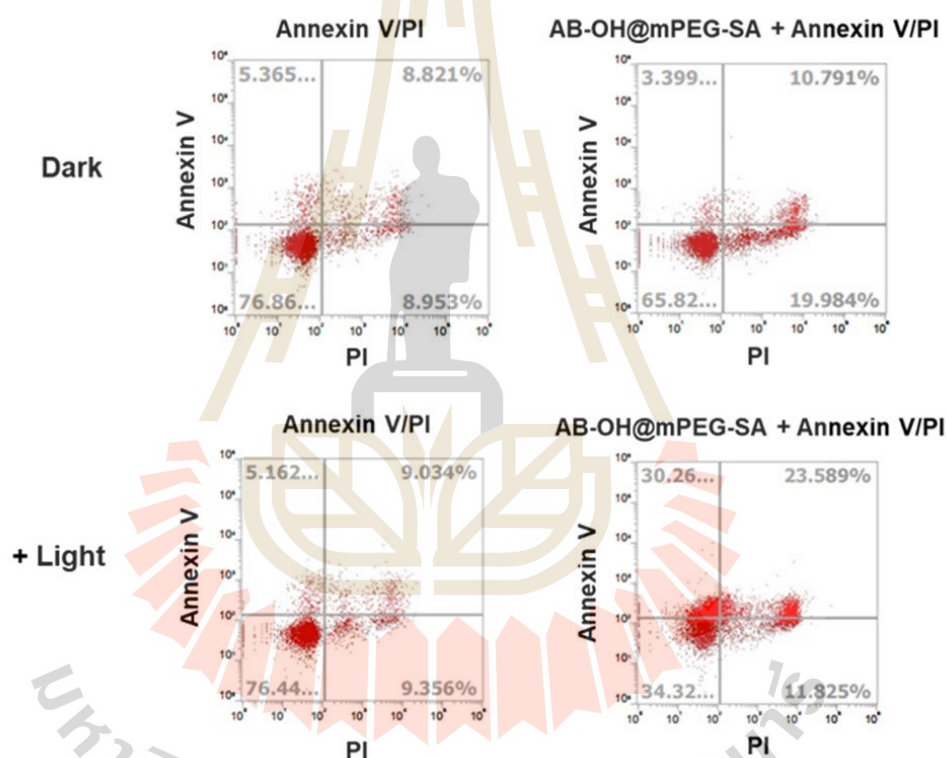


Figure 4.11 Flow cytometry Annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis analyses under dark (top) and 15 min light irradiation (bottom).

To investigate the photodynamic-assisted cancer cell apoptosis by our NPs, evaluation of apoptosis via flow cytometry relies on annexin V-FITC, a vital dye targeting phosphatidylserine early in the process. Additionally, propidium iodide (PI) is used concurrently to confirm nuclear changes in late-stage apoptosis, indicative of

heightened membrane permeability. In this study, we further used fluorescence-activated cell sorting (FACS) to assess photodynamic-assisted cancer cell apoptosis. **Figure 4.11** demonstrated that when the light was on, the treated cells with **AB-OH@mPEG-SA** induced 30.26% early and 23.59% late apoptosis. This rate is considerably higher than those of cells treated with i) dark (9%), ii) NPs without light irradiation (10%), and iii) light alone (9%). These findings indicated that the dye, even after forming NPs, retained its light activity and its ability to destroy cancer cells through photodynamic mechanisms.

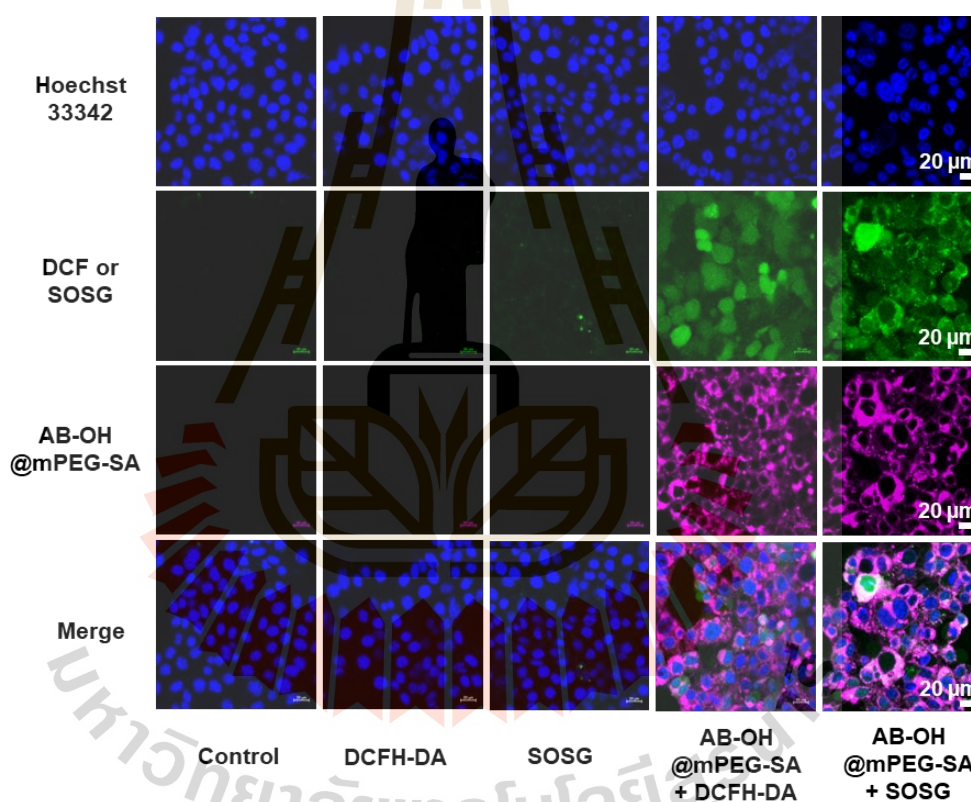


Figure 4.12 Intracellular ROS and SOSG detection in 4T1 cells under different conditions; non-treated cells (1st column); DCFH-DA (2nd column); SOSG (3rd column); AB-OH@mPEG-SA and DCFH-DA (4th column); AB-OH@mPEG-SA and SOSG (last column). Scale bar = 20 μm.

To verify whether an oxidative species generated upon light illumination played an important role in the phototoxicity of **AB-OH@mPEG-SA** NPs, the intracellular reactive oxygen species (ROS) and singlet-oxygen generation assay were conducted. To detect ROS, 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Aranda et al., 2013) was employed, which would be quickly oxidized by intracellular ROS, leading to the formation of a bright green fluorescence 2',7'-dichlorofluoresce (DCF). Additionally, we also investigated the generation of $^1\text{O}_2$ within the cells using singlet-oxygen sensor green (SOSG) (Gollmer et al., 2011). After the uptake of prepared NPs with 4T1 cells for 24 h, the cells were co-stained with DCFH-DA or SOSG followed by 660 nm red light illumination (8.7 mW cm^{-1}) for 15 minutes before observing under a confocal microscope. Green fluorescence signals were detected in only cells carrying **AB-OH@mPEG-SA** NPs with DCFH-DA or SOSG subjected to light (Figure 4.12), indicating increasing $^1\text{O}_2$ or ROS were produced upon red light irradiation. On the other hand, this phenomenon was not observed in the other control groups (non-treated cells, DCFH-DA only, and SOSG only). Therefore, **AB-OH@mPEG-SA** can produce ROS including $^1\text{O}_2$ to induce cell death via the apoptosis process.

4.4.7 *In vitro* studies on three-dimensional (3D) breast cancer tumor models

Furthermore, we also evaluated the effectiveness of **AB-OH@mPEG-SA** NPs in 3D tumor models before moving to *in vivo*. The accumulation of prepared NPs in tumor spheroids was carried out using confocal microscopy. As demonstrated in Figure 4.13A, the 4T1 spheroids exhibited enhanced fluorescence from **AB-OH@mPEG-SA** NPs with increasing incubation time, revealing bright signals after the first 3 h of incubation, whereas the bright signal was seen when free **AB-OH** accumulated within the spheroid structure after 12 h incubation as provided in supporting information, which agrees well with the internalization assay in two-dimensional (2D) cell culture.

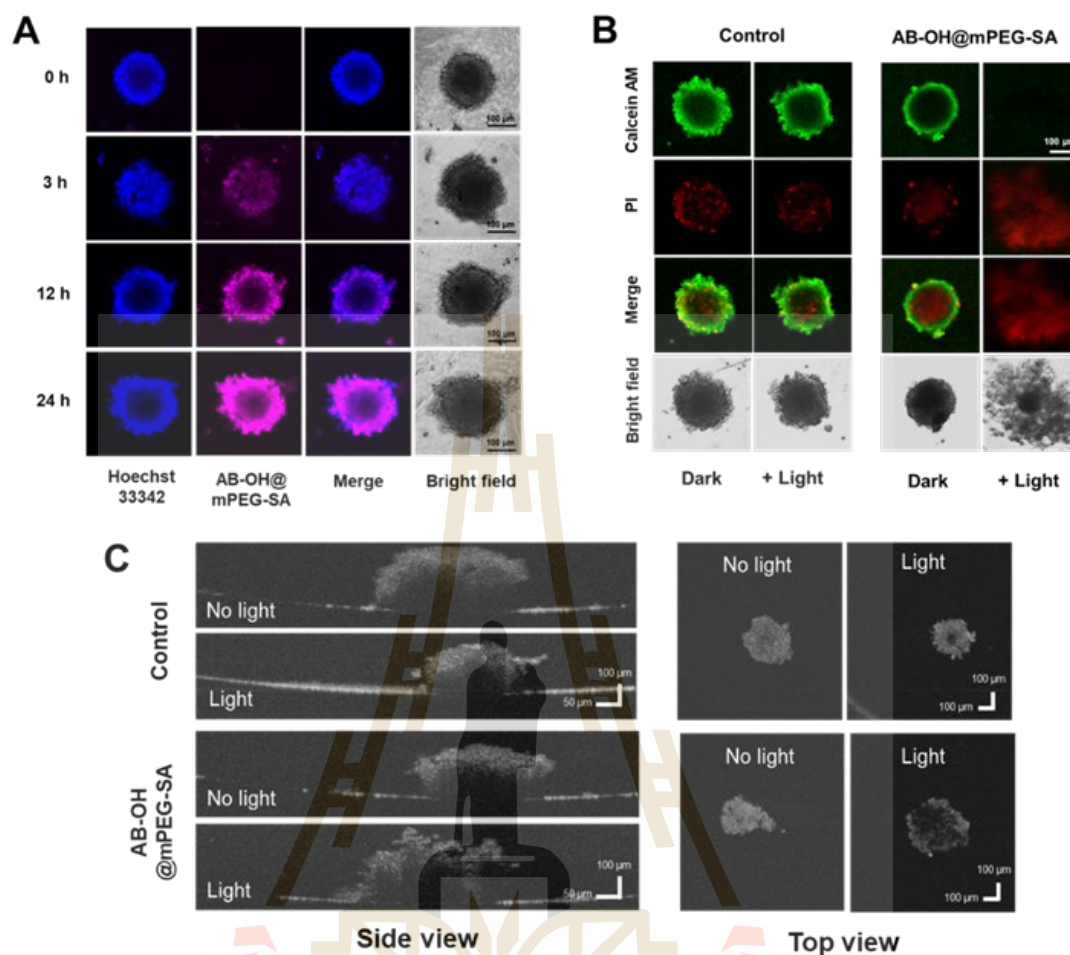


Figure 4.13 *In vitro* studies on 3D breast cancer tumor models. (A) Confocal images of 4T1 spheroids incubated with AB-OH@mPEG-SA (2 μ M) for various time points (0, 3, 12, and 24 h). Ex: 408 nm for Hoechst 33342 and 643 nm for AB-OH; Em: 425–475 nm for Hoechst 33342 and 663–738 nm for AB-OH. Scale bars = 100 μ m. (B) Live/dead cell imaging of 4T1 spheroid cells incubated with AB-OH@mPEG-SA (5 μ M) under 30 min light irradiation and dark conditions. Ex: 488 nm for Calcein AM and 561 nm for PI; Em: 500–550 nm for Calcein AM and 570–620 nm for PI. Scale bars = 100 μ m. (C) 3D imaging using frequency domain optical coherence tomography (FD-OCT) of AB-OH@mPEG-SA upon light irradiation and dark conditions using non-treated cells as a control. Scale bars = 100 μ m.

To prove the anticancer activity of **AB-OH@mPEG-SA** NPs towards tumor spheroids, we then performed live/dead viability/cytotoxicity assay in the 3D *in vitro* models. As shown in **Figure 4.13B**, the 4T1 spheroids treated with **AB-OH@mPEG-SA** NPs under light irradiation were stained with red fluorescence signals from PI, demonstrating significant phototoxicity. In contrast, cells treated with the probe in the absence of light irradiation exhibited mostly green fluorescence, implying that most of the spheroids were alive. Moreover, the **AB-OH@mPEG-SA** NPs displayed the significant destruction of the 4T1 spheroids, when compared to the spheroid treated with free **AB-OH** as illustrated in supporting information. These live/dead co-staining results are consistent with the photocytotoxicity assay in 2D cell culture.

Furthermore, optical coherence tomography (OCT) was employed to monitor the response after treatment on tumor structures. OCT is a noninvasive imaging technology that can perform high-speed and high-resolution depth-cross-sectional imaging of biological tissue (Aumann, Donner, Fischer, and Müller, 2019; Vakoc, Fukumura, Jain, and Bouma, 2012). During tumor growth and anticancer drug therapy, OCT has been utilized to monitor tumor burden (Snuderl et al., 2013), disclose wide-field tumor vascular morphology, noninvasively monitor lymphangiogenesis and lymph artery malfunction, and distinguish viable and nonviable tumor compartments (Nam, Vakoc, Blauvelt, and Chico-Calero, 2015). As seen in **Figure 4.13C**, the untreated spheroid showed good spherical morphology both in the presence or absence of light conditions, whereas only the spheroid treated with **AB-OH@mPEG-SA** NPs demonstrated extreme damage after being exposed to light for 30 min. These results confirmed the phototoxicity of NPs toward tumor spheroids.

4.5 Conclusion

In summary, we designed and developed a novel aza-BODIPY-based organic molecule (**AB-OH**) with phototherapeutic properties. The **AB-OH@mPEG-SA** NPs was obtained by conjugation of **AB-OH**, with a biocompatible polymer, mPEG-SA, and directly self-assembly to form stable nanoparticles. The prepared NPs appeared spherical with average hydrodynamic sizes of 170 nm. Under the irradiation of a 660 nm red LED lamp, the NPs generated enough amounts of $^1\text{O}_2$ to damage the cancerous cells in both the solution and the intracellular matrix. Additionally, **AB-OH@mPEG-SA** NPs exhibited great biocompatibility and phototoxicity against 4T1 cells both in 2D and 3D cell cultures. Furthermore, additional proof of the effectiveness of the NPs has been tested in the murine model.

4.6 References

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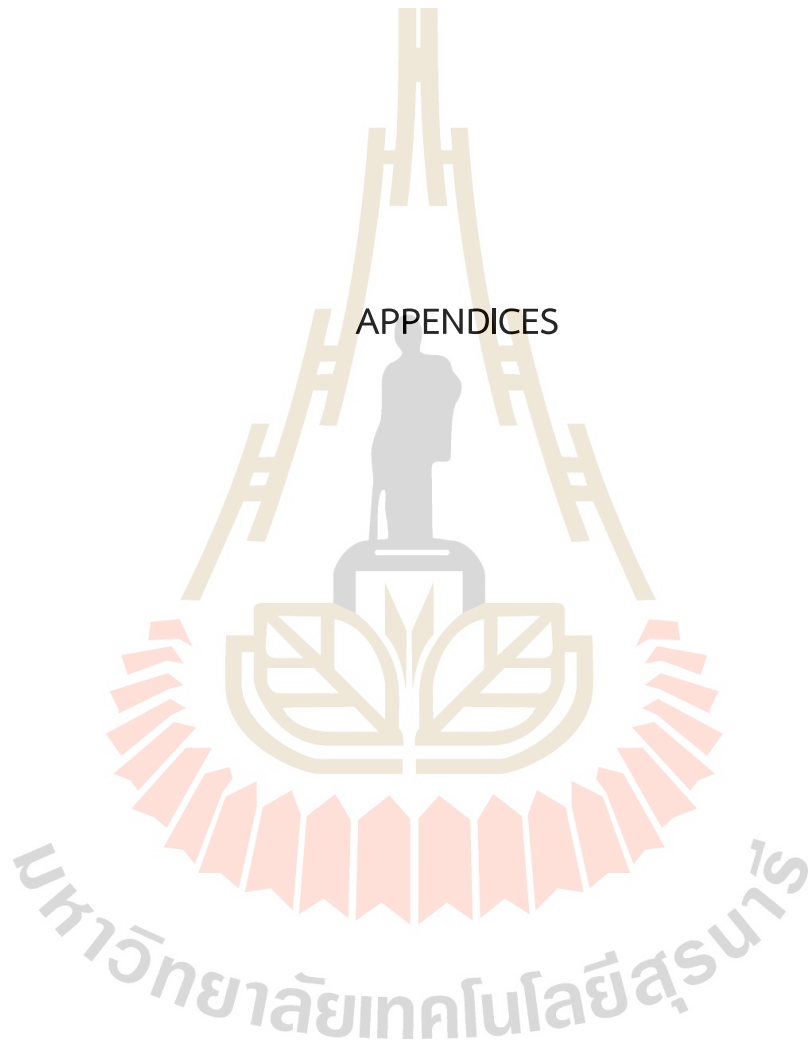
CHAPTER V

CONCLUSIONS

In summary, we successfully developed two nanosystems using different photosensitizers—cyanine and Aza-BODIPY dyes—for effective photodynamic therapy (PDT) and cancer diagnosis. For the cyanine-based nanoparticles, we combined the cyanine dye (**I₂-IR783**) with the chemotherapeutic agent (MTX) to create stable nanoparticles that exhibited favorable Vis-NIR absorption and emission properties for PDT and fluorescence imaging. Upon 805 nm LED light exposure, the **I₂-IR783/MTX@NPs** efficiently produced singlet oxygen ($^1\text{O}_2$), triggering significant cytotoxicity in cancer cells, with low concentrations showing efficacy. These NPs also demonstrated potent anticancer effects in 3D tumor spheroids and were monitored using optical coherence tomography (OCT), confirming their potential for synergistic photo-chemotherapy.

In addition, we developed a novel Aza-BODIPY-based nanoparticle (**AB-OH@mPEG-SA**), which exhibited strong phototherapeutic properties and excellent biocompatibility. These nanoparticles, when irradiated with a 660 nm red LED lamp, generated sufficient $^1\text{O}_2$ to effectively target cancer cells both in solution and within cells. The **AB-OH@mPEG-SA** NPs showed significant phototoxicity in 2D and 3D cell cultures, and their efficacy was further confirmed in murine models. These findings highlight the potential of both nanoparticle systems as promising candidates for cancer phototherapy.

APPENDICES



APPENDIX A

SUPPORTING INFORMATION

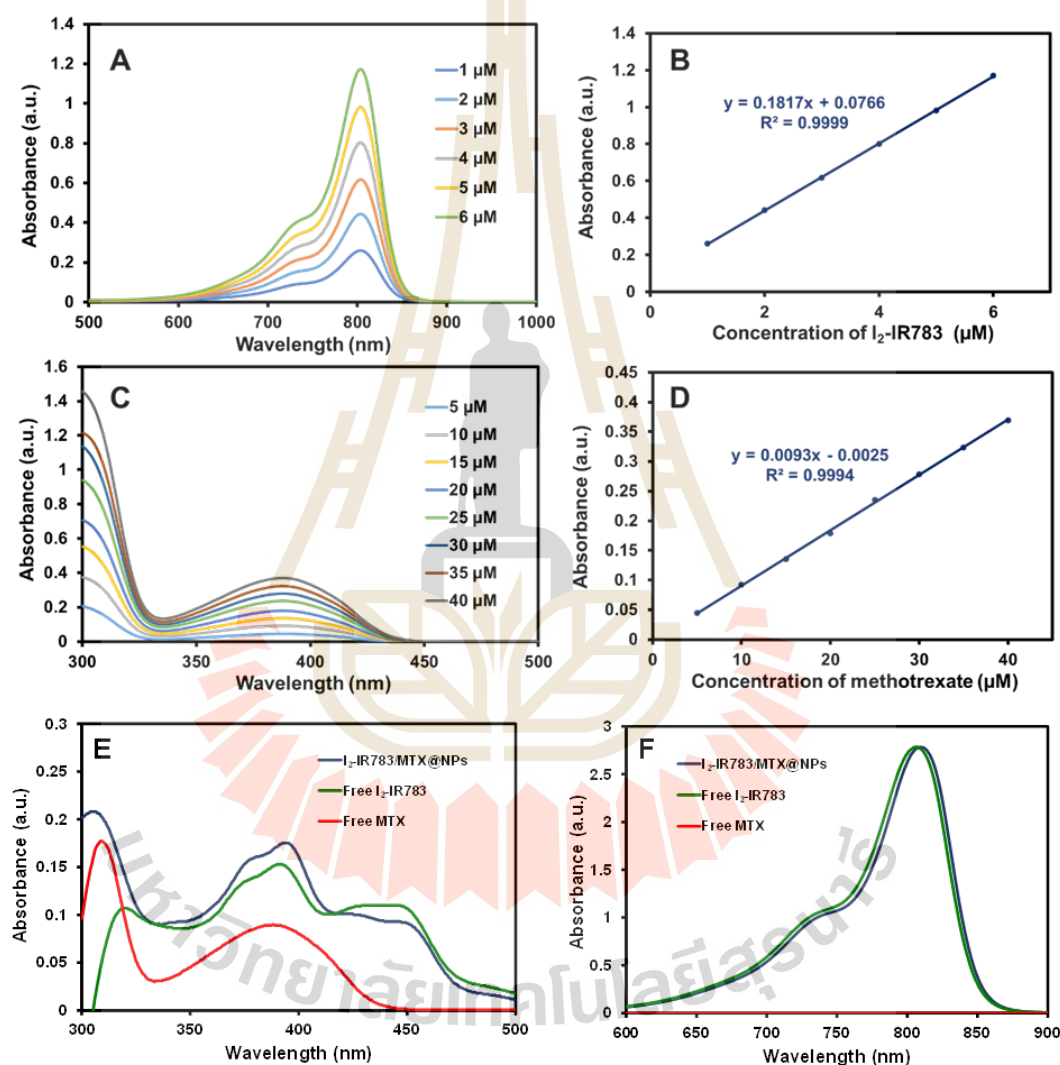


Figure S1 (A) Absorption spectra of free I_2 -IR783 in various concentrations. (B) Calibration curve of free I_2 -IR783. (C) Absorption spectra of methotrexate drug in various concentrations. (D) Calibration curve of methotrexate. (E) and (F) Overlay absorption spectra of free I_2 -IR783, I_2 -IR783/MTX@NPs, and free MTX.

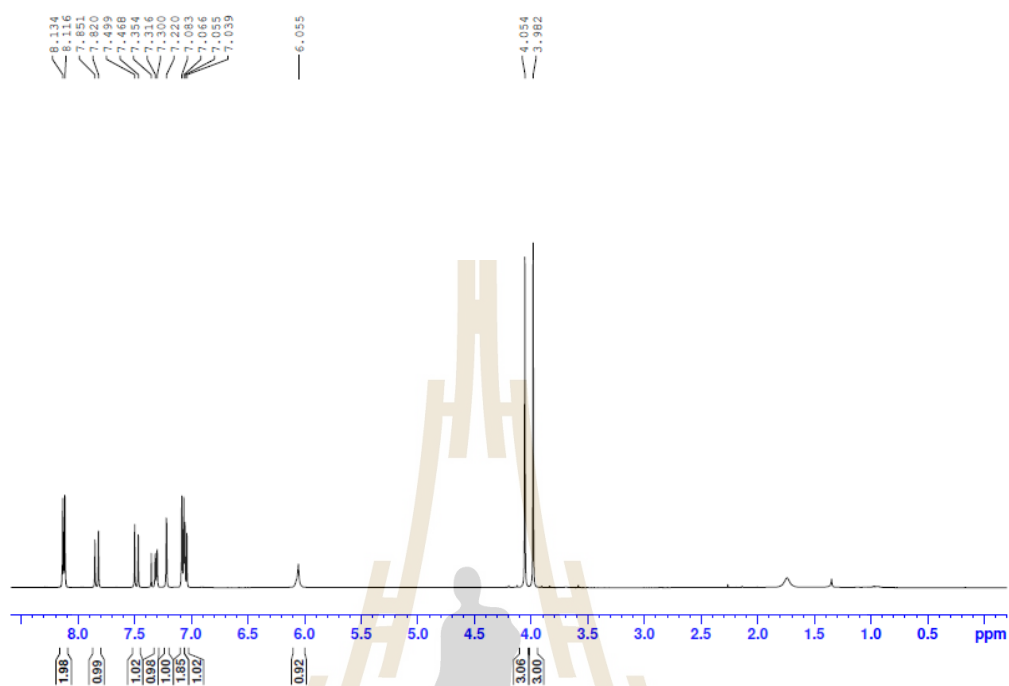


Figure S2 ¹H NMR spectrum of compound 1 in *d*-chloroform.

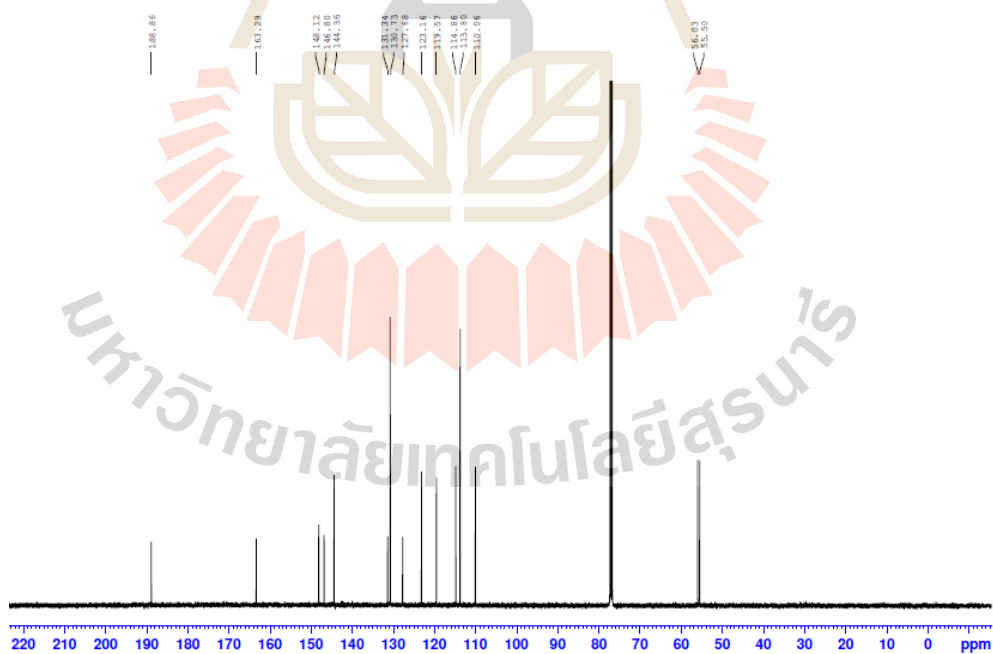


Figure S3 ¹³C NMR spectrum of compound 1 in *d*-chloroform.

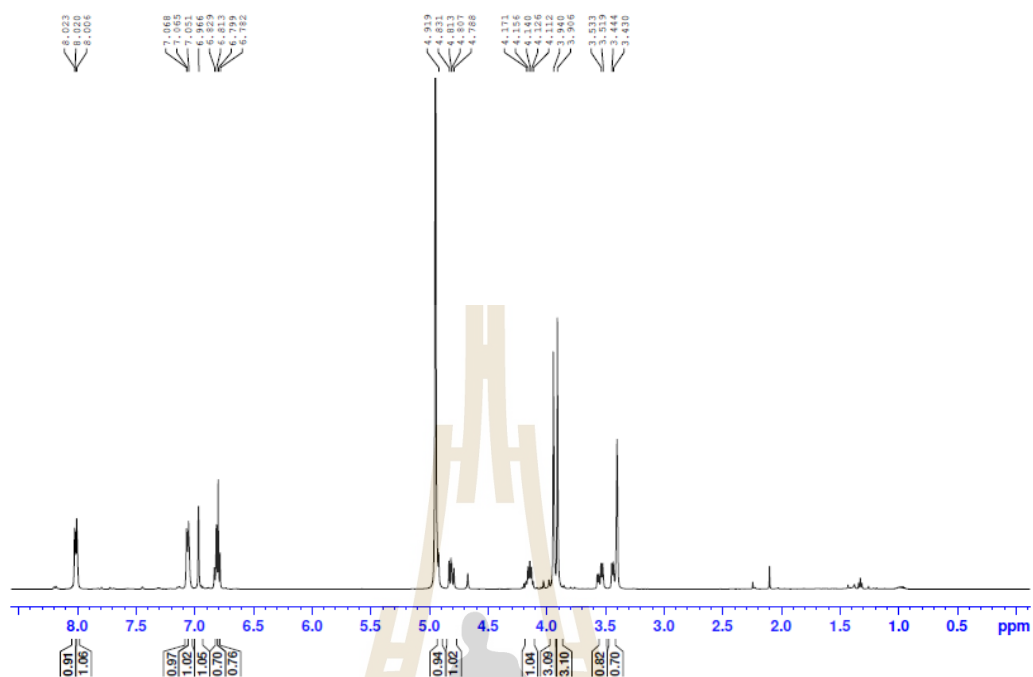


Figure S4 ¹H NMR spectrum of compound 2 in *d*₄-methanol.

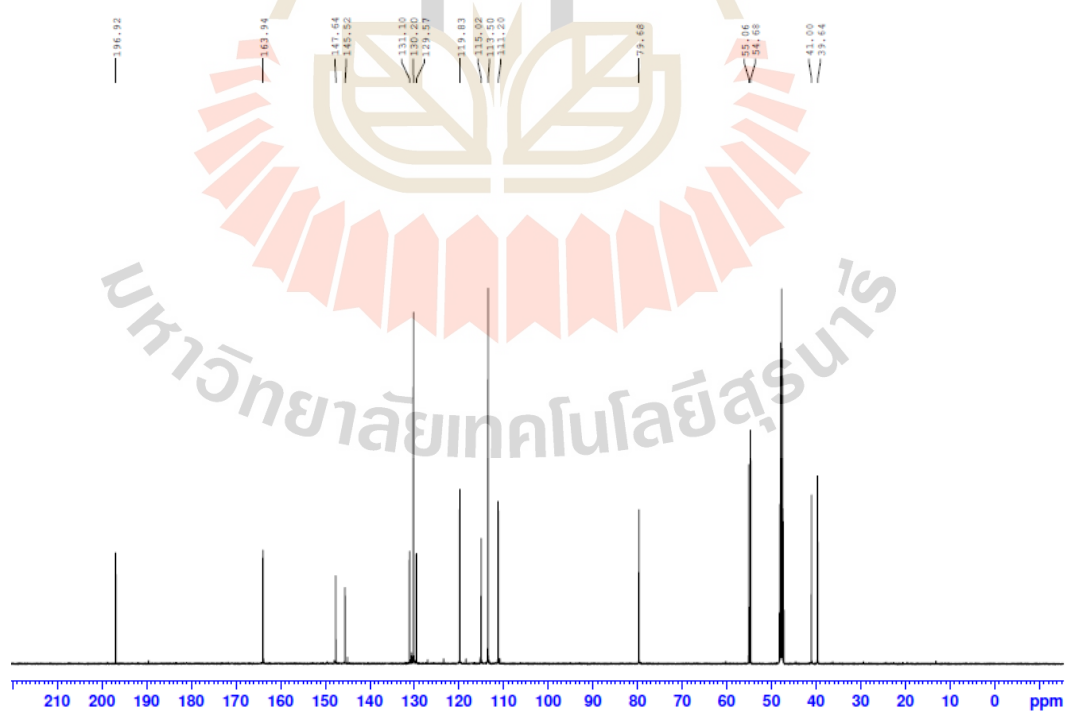


Figure S5 ¹³C NMR spectrum of compound 2 in *d*₄-methanol.

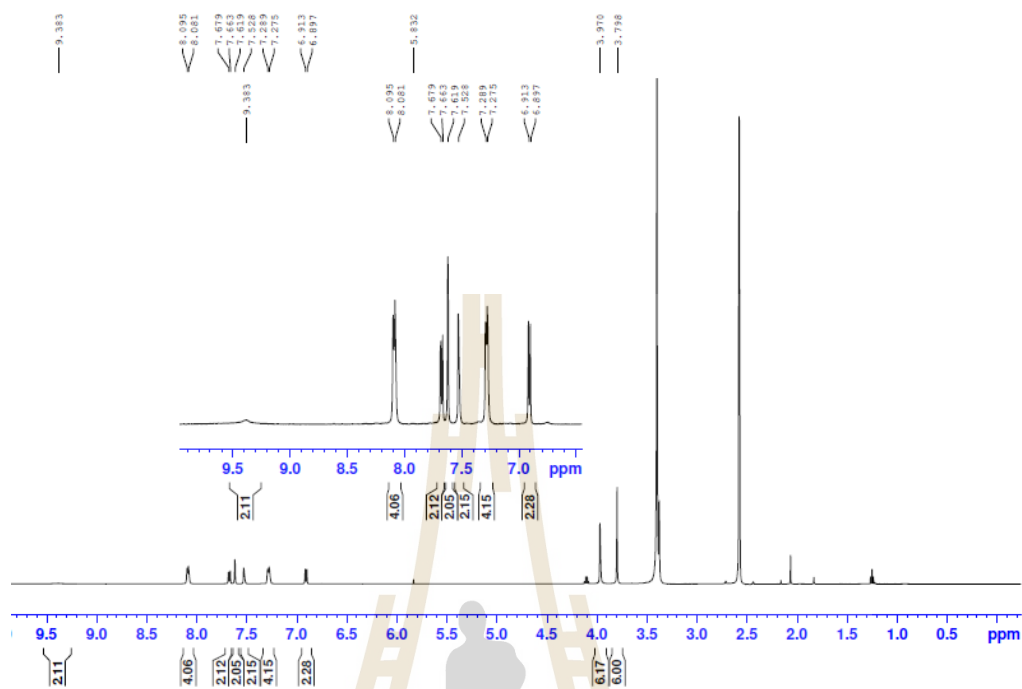


Figure S6 ¹H NMR spectrum of compound 3 in *d*₆-DMSO.

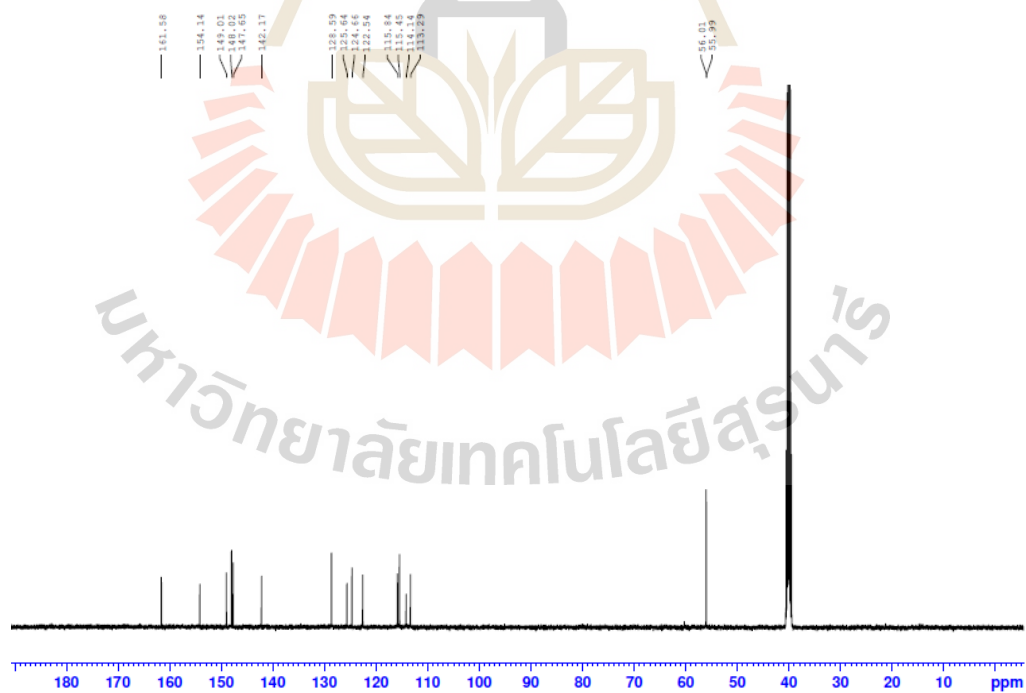


Figure S7 ¹³C NMR spectrum of compound 3 in *d*₆-DMSO.

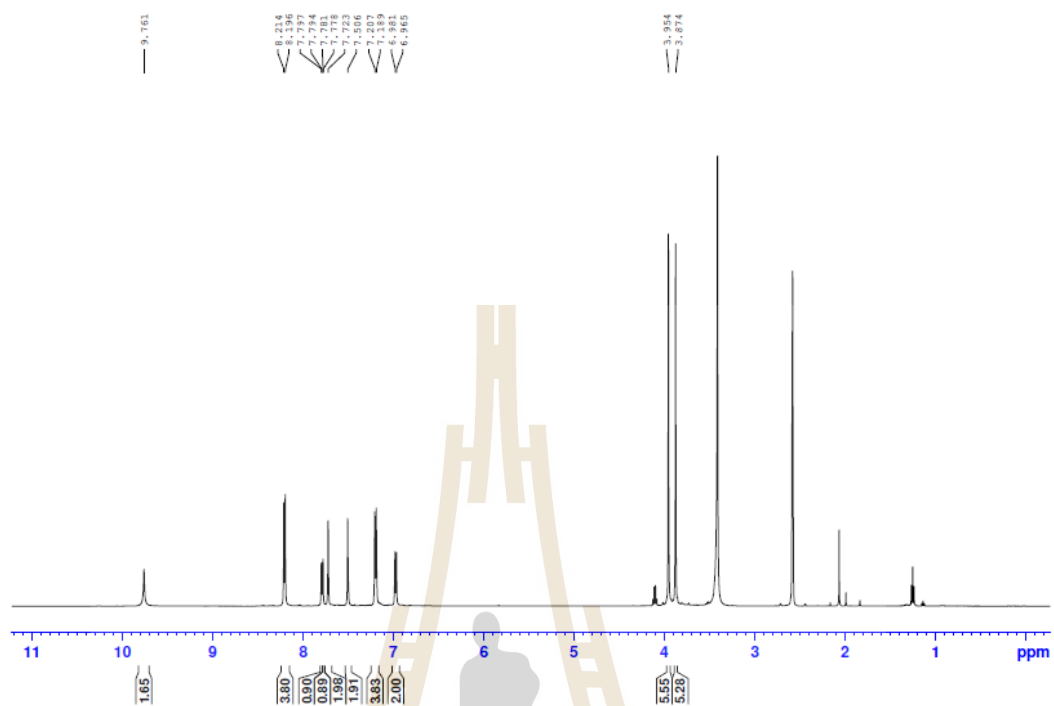


Figure S8 ¹H NMR spectrum of compound 4 in *d*₆-DMSO.

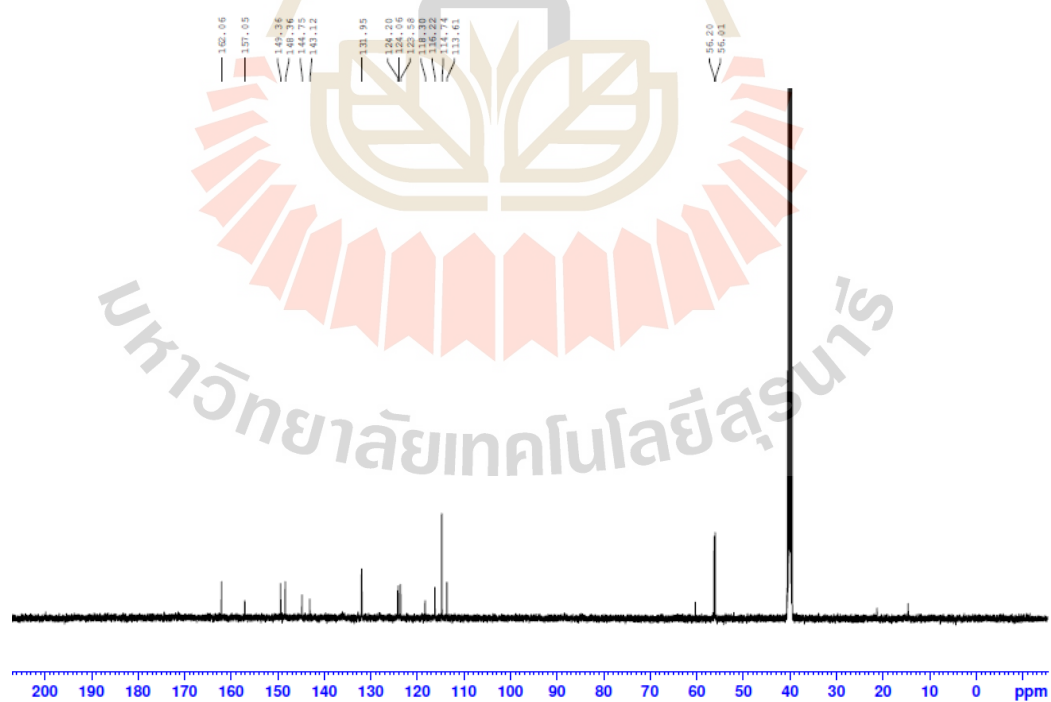


Figure S9 ¹³C NMR spectrum of compound 4 in *d*₆-DMSO.

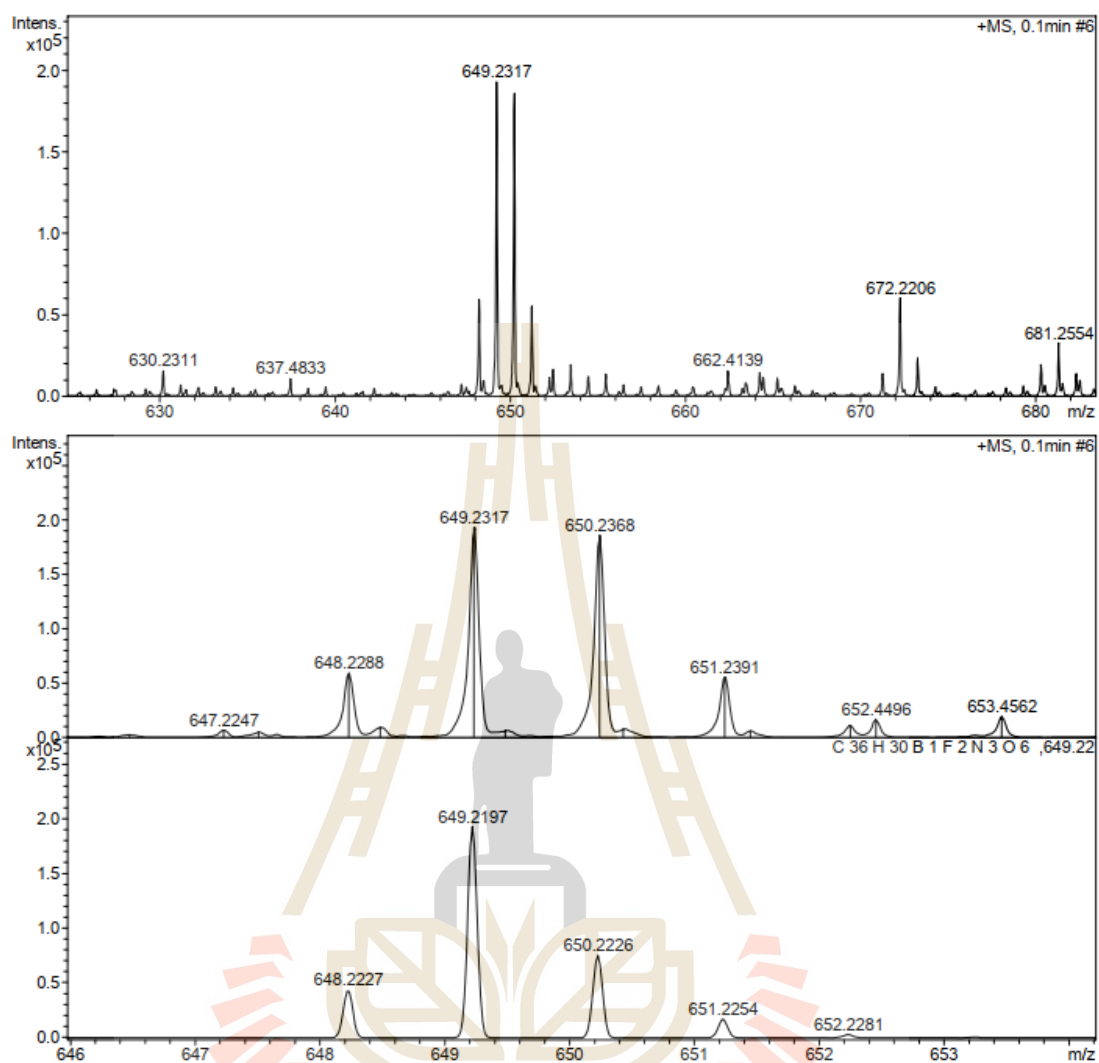


Figure S10 HRMS of AB-OH.

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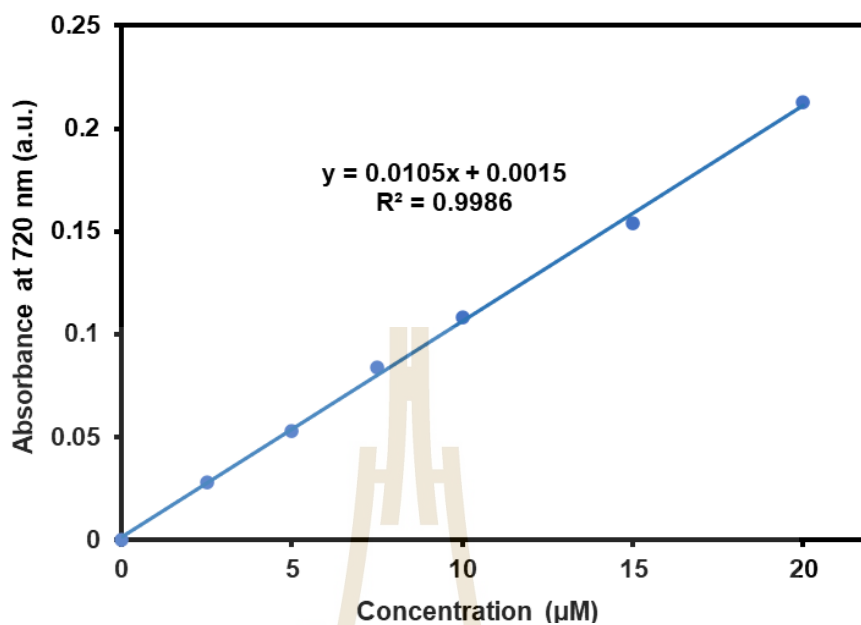


Figure S11 Calibration curve of AB-OH in DMSO.

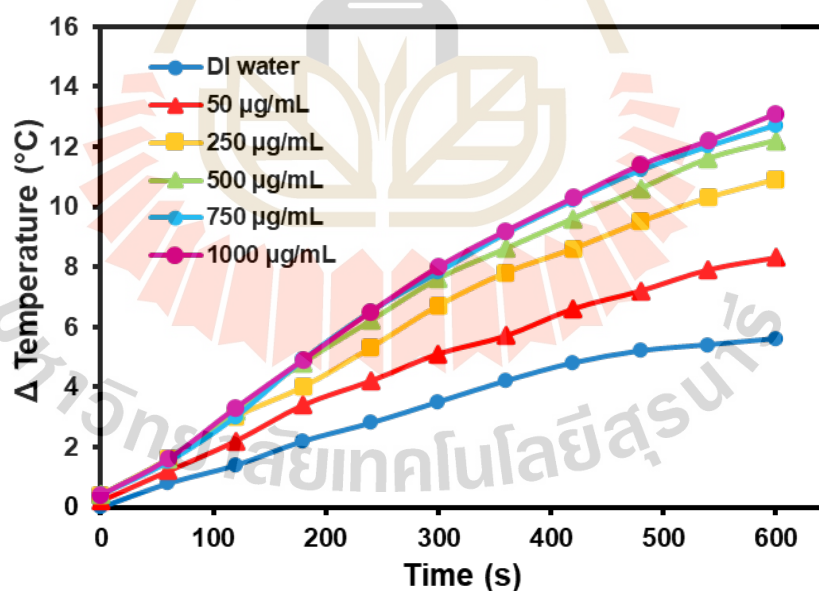


Figure S12 Temperature rise curves of AB-OH@mPEG-SA solutions at different NP contents (0, 50, 250, 500, 750, and 1,000 $\mu\text{g/mL}$) in DI-water after being irradiated by a 660 nm red LED lamp at a power density of 60 mW cm^{-2} .

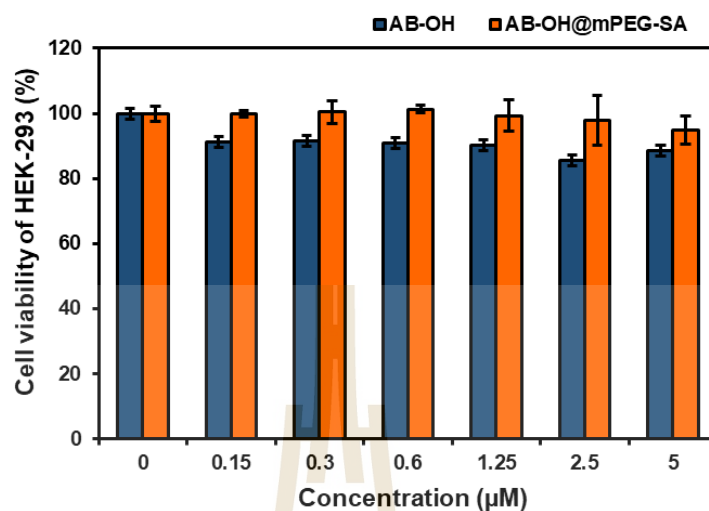


Figure S13 The relative cell viability of HEK-293 cells with various concentrations of AB-OH and AB-OH@mPEG-SA NPs treatment (0-5 μM) under dark conditions.

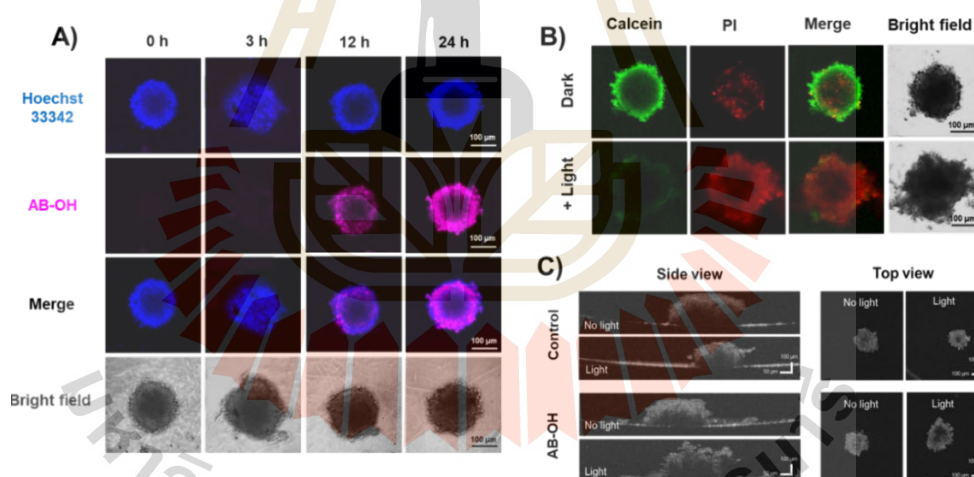


Figure S14 (A) Confocal images of 4T1 spheroids incubated with free AB-OH (2 μM) for various time points (0, 3, 12, and 24 h). Scale bars = 100 μm . (B) Live/dead cell imaging of 4T1 spheroid cells incubated with AB-OH (5 μM) under 30 min light irradiation and dark conditions. Scale bars = 100 μm . (C) 3D imaging using frequency domain optical coherence tomography (FD-OCT) of AB-OH (5 μM) upon light irradiation and dark conditions using non-treated cells as a control. Scale bars = 100 μm .

APPENDIX B

PUBLICATIONS

B.1 List of publications

First author and Co-first authors

Muangsoapa, P., Chansaenpak, K., Kampaengsri, S., Saetiew, J., Noisa, P., Meemon, P., Kamkaew, A. (2023). Hybrid Cyanine/Methotrexate Nanoparticles for Synergistic PDT/Chemotherapy of Breast Cancer. *ACS Appl. Bio Mater.*, 6, 2, 603–614. doi.org/10.1021/acsabm.2c00893.

Duangkamol, C., **Muangsoapa, P.**, Rattanopas, S., Wongsuwan, P., Khrootkaew, T., Chueakwon, P., Niamnont, N., Chansaenpak, K., Kamkaew, A. (2023). Polarity and viscosity-sensitive fluorescence probes for lipid droplet imaging in cancer cells. *Dyes Pigm.*, 216, 111365. doi.org/10.1016/j.dyepig.2023.111365.

Kampaengsri, S., **Muangsoapa, P.**, Pangjantuk, A., Chansaenpak, K., Lai, R.-Y., Noisa, P., Kamkaew, A. (2024). Cannabidiol and Aza-BODIPY Coencapsulation for Photodynamic Therapy Enhancement in Liver Cancer Cells. *ACS Appl. Bio Mater.*, 7, 6, 3890-3899. doi.org/10.1021/acsabm.4c00239.

Co-authors

Wiriya, N., Wangngae, S., **Muangsoapa, P.**, Chueakwon, P., Khrootkaew, T., Chansaenpak, K., Ngernsounghern, A., Lai, R.-Y., Kamkaew, A. (2024). Versatile iodinated styryl dyes: Promising probes for detection with dual anti-cancer and anti-bacterial properties. *J. Mol. Struct.*, 1322, 140512. doi.org/10.1016/j.molstruc.2024.140512.

Saiyasombat, W., **Muangsoapa, P.**, Khrootkaew, T., Chansaenpak, K., Piyanuch, P., Sapermsap, M., Sangtawesin, S., Kamkaew, A. (2024). Halogenated BOIMPYs and Their Efficiency in Photodynamic Therapy. *ChemPhotoChem.* 8 (10). doi.org/10.1002/cptc.202400109.

Khaikate, O., **Muangsoapa, P.**, Piyanuch, P., Khrootkaew, T., Wiriya, N., Chansaenpak, K., Sukwattanasinitt, M., Kamkaew, A. (2024). Asymmetric heptamethine cyanine dye for viscosity detection and photodynamic therapy. *J. Photochem. Photobiol. A: Chem.*, 453, 115659. doi.org/10.1016/j.jphotochem.2024.115659.

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Khaikate, O., Pewklang, T., Khrootkaew, T., Chansaenpak, K., **Muangsoapa, P.**, Kuhakarn, C., Kamkaew, A. (2023). Unraveling the Photophysical Characteristics and Biological Applications of Vinyl Sulfones as Viscosity Sensors. *RSC Adv.*, 13 (24), 16671-16677. doi.org/10.1039/d3ra02354k.

Kampaengsri, S., Chansaenpak, K., Pewklang, T., **Muangsoapa, P.**, Cairns, J. R. K., Lai, R.-Y., Kamkaew, A. Quercetin Nanoparticle-Based Hypoxia-Responsive Probe for Cancer Detection. (2023) *ACS Appl. Bio Mater.*, 6 (4), 1546–1555. doi.org/10.1021/acsabm.2c01063.

Wet-osot, S., Pewklang, T., Duangkamol, C., **Muangsoapa, P.**, Ngivprom, U., Chansaenpak, K., Ngernsounghem, A., Sritangos, P., Chudapongse, N., Lai, R.-Y., Kamkaew, A. (2022). N-Tosylindole-coumarin with high fluorescence quantum yield and their potential applications. *J. Mol. Struct.*, 1260, 132840. doi.org/10.1016/j.molstruc.2022.132840.

CURRICULUM VITAE

Name Prapassara Muangsopa

Date of Birth March 7th, 2000, at Nakhon Ratchasima, Thailand

Education

- 2022 – present Master of Science in Chemistry (International Program),
Suranaree University of Technology, Thailand (expected 2025)
- 2018 – 2022 Bachelor of Science in Chemistry, Suranaree University of
Technology, Thailand (1st Class Honors)

Research Experiences

- January – June 2025 Centre for Inflammation Research, Institute for Regeneration
and Repair, The University of Edinburgh, Edinburgh, Scotland,
UK, under the supervision of Professor Marc Vendrell Escobar.
- July – August 2023 Taiwan International Graduate Program (TIGP) Internship,
Institute of Atomic and Molecular Sciences, Academia Sinica,
Taipei, Taiwan, under the supervision of Dr. Ching-Wei Lin.

Awards

- 2025 Thai Student Representative for the 74th Lindau Nobel Laureate Meeting
(Chemistry), Lindau, Germany.
- 2024 Best Poster Presentation Award in the Science Postgrad Annual Research
Conference (SPARC 2024).
- 2022 Dr. Tab Nilanidhi's Award for the highest distinction GPA in B.Sc. Chemistry.
- 2022 Gold Prize Award from Young Rising Stars of Science 2022.
- 2018 DPST Scholarship for study in the field of Chemistry through M.Sc.