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Synthesis and cell-imaging applications of water-soluble BODIPY dyes

Supervisor Prof. Kim Ho-Joong

This thesis is submitted in partial fulfillment
of the requirements for the
Master of Science in Chemistry.

April 2019

Chosun University Graduate School

Department of Chemistry

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Badon, Isabel Wen, Joomin Lee, Temmy Pegarro Vales, Byoung Ki Cho, and Ho-Joong Kim. "Synthesis and photophysical characterization of highly water-soluble PEGylated BODIPY derivatives for cellular imaging." Journal of Photochemistry and Photobiology A: Chemistry 377 (2019): 214-219.

Vales, Temmy Pegarro, Isabel Wen T. Badon, and Ho-Joong Kim. "Multi-Responsive Hydrogels Functionalized with a Photochromic Spiropyran-Conjugated Chitosan Network." Macromolecular Research 26, no. 10 (2018): 950-953.

Lee, Cheol Woo, Isabel Wen Badon, Boram Kim, Geun-Chang Ryu, and Ho-Joong Kim. "Fabrication of Spiropyran-functionalized Photochromic Hydrogel Lenses." 조선자연과학논문집 11, no. 1 (2018): 39-43.

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ABSTRACT

Synthesis and cell-imaging applications of water-soluble BODIPY dyes

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A series of poly(ethylene glycol) (PEG)-modified water-soluble boron dipyrromethene (BODIPY) dyes (EtBODPEG and BOD-PEG) were synthesized and their photophysical properties in various solutions were investigated. The presence of ethyl groups at the 2,6-positions of the BODIPY core in EtBOD-PEG resulted in bathochromic shifts in both the absorption and emission spectra relative to those of the unsubstituted BOD-PEG. Importantly, bulky di-branched PEG chains were introduced at the meso position of the BODIPY core, leading to enhanced solubilities and high fluorescence quantum yields in aqueous solutions. The water-soluble BODIPY dyes were explored for their applicability in fluorescent bio-imaging using MCF-7 human breast cancer cells. The dyes were cell-permeable and were concentrated in cellular cytoplasm area, suggesting that they have potential applications as bio-imaging agents.

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Chapter 1: Fluorescence

1. Introduction

1.1. Basic Principles of Fluorescence

When a molecule emits light of a certain wavelength upon irradiation by light of a shorter wavelength, that molecule is said to be fluorescent. Fluorescence is commonly produced by single-photon excitation, wherein a number of molecules excited by an appropriate wavelength of light absorb enough photons to promote an electron from the ground-state energy level (S_0)to an excited-state energy level(S_1).[1] When this excited electron relaxes back to the ground state energy level, it emits a photon of lower energy than the absorbed one. This transition from the excited state (S_1)to the ground state (S_0) is characterized by i) fluorescence intensity as a function of wavelength, ii) fluorescence quantum yield, and iii) fluorescence lifetime.

(1) $$S_0 + hv_{ex} \rightarrow S_1$$

(2) $$S_1 \rightarrow S_0 + hv_{em} + heat$$

Where:

S_0-ground-state energy level

S_1-first electronically excited energy level

hv - photon energy

The process of fluorescence is better understood using a Jablonski energy diagram as shown in Figure 1. When an electron is excited, several processes compete to de-excite it to the ground state, which are fluorescence, internal conversion or vibrational relaxation, intersystem crossing from singlet state to triplet state and phosphorescence. Each of these processes is can occur at certain probabilities associated with decay rate constants (k). Hence, the weaker the alternative pathways, the higher the relaxation *via* fluorescence. Interestingly, even if the fluorophore is excited into different singlet state energies (i.e. S1, S2, S3, etc.), thermal relaxation will eventually bring the fluorophore to the first electronic state (S1) where emission can occur. This is the reason behind the independence of the emission spectrum from the excitation wavelength.[2]

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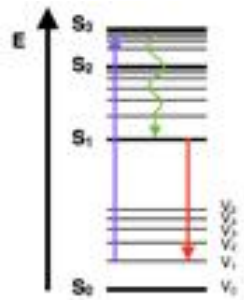

Figure 1. Jablonski energy diagram showing absorbance (purple), vibrational relaxation (green) and fluorescence (orange). Source: chem.libretexts.org

The fluorescence spectrum can be used to characterize a molecule. The intensity, position of the emission wavelength and fluorescence lifetime are some of the properties of a molecule that can be gathered through fluorescence emission. There are intrinsic properties unique for each fluorophore and can be modified by the environment.

1.2. Stokes shift

The Stokes shift, the wavelength difference between absorbance and emission peak maxima, is an important parameter for fluorescent dyes.[3] This shift to longer wavelength was first observed by Sir George Stokes in 1852. Stokes shift stems from the fact that at room temperature, ground state fluorophores are generally in the lowest vibrational level of the first electronic state as explained previously. With E_a and E_{em} to describe absorption and emission energies, respectively, it can be shown that:

(3) $$E_a = hc/\lambda_a$$

(4) $$E_{em} = hc/\lambda_{em}$$

Due to the competitive radiative and non-radiative processes,

(5) $$E_{em} < E_a$$

(6) Hence, $$\lambda_{em} > \lambda_a$$

On some applications, a large Stokes shift is favored to minimize the overlap between the absorption and emission spectra.[4] The lower the Stokes shift, the greater the overlap will be between the absorption and emission spectra, resulting to loss due to reabsorption of emitted

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photons. Hence, to minimize reabsorption of emitted photons, Stokes shifts greater than 80 nm are desirable.[5]

1.3. Inner Filter Effect

Another consideration to make when measuring fluorescence is the effect of the optical density of the sample on the observed signal. As much as fluorescence is a popular method to studying various compounds, a crucial obstacle in using this property lies in the nonlinear dependence of fluorescence intensity to the concentration of the fluorescent compounds. This effect is called the "inner-filter effect" (IFE) and results to incorrect use of the method even by experienced researchers.[6] The IFE is classified into the primary inner filter effect (PIFE) and the secondary inner filter effect (SIFE). The PIFE is the attenuation of the excitation beam as it reaches each subsequent layer of the solution due to the absorption of the chromophores, whereas the SIFE is the reabsorption of emitted photons by other surrounding chromophores.[7] However, this effect can be minimized by diluting the solution or by incorporating a correction factor in the mathematical model of filter effects.[8]

1.4. Fluorescence lifetime

Fluorescence lifetime is defined as the time needed to reduce a population of excited fluorophores through fluorescence and other non-radiative processes.[9] This time varies from nanoseconds (10^{-9}s)to(10^{-12}s)and is shown by the equation:

(7)
$$\tau_f = \frac{1}{k} = \frac{1}{k_r + k_{ISC} + k_I}$$

Where:

τ_f = fluorescence lifetime

The equation for fluorescence lifetime accounts for both radiative and non-radiative processes involved in the relaxation of the electron.

Fluorescence lifetime is an intrinsic property of a fluorophore and is independent on initial perturbation conditions, duration of light exposure, one- or multi-photon excitation, and photobleaching. Moreover, it is not affected by fluorescence intensity and fluorophore concentration. Since fluorescence lifetime is associated with energetically unstable states, it can be sensitive to various internal and external factors such as fluorophore structure, temperature, polarity and presence of fluorescence quenchers.[9]

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1.5. Fluorescence quantum yield

After molecules in the ground state absorb energy and electrons are promoted to an excited state (S_n),different relaxation processes, including fluorescence, will compete.[10] The rate constant (k) of the excited state includes the kinetic constants of the competing relaxation processes:

(8) $$k = k_r + k_{ISC} + k_I$$

The fluorescence quantum yield (ϕ_F)is a measure of the number of photons emitted radiatively (k_r) over the total absorbed photons. Essentially, ϕ_F determines the efficiency of the conversion of absorbed light into emitted light.[11]

(9) $$\varphi_F = \frac{emitted\ photons}{absorbed\ photons} = \frac{k_r}{k_r + k_{ISC} + k_I}$$

Experimentally, the fluorescence quantum yield is measured by comparing the fluorescence intensity of the molecule with that of reference standard with a known quantum yield.

(10) $$\varphi_2 = \frac{OD_1 * \sum F_2}{OD_2 * \sum F_1}\ \varphi_1$$

Where:

F_2-fluorescence intensity of the molecule with unknown quantum yield,

F_1-fluorescence intensity of the reference molecule with quantum yield ϕ_1.

Therefore, in order to measure the quantum yield of a compound, one must measure the optical densities and fluorescence intensities of the fluorophore and reference compound under the same conditions and environment.

This method of determining the quantum yield using a reference compound is called the relative fluorescence quantum yield. Recently, absolute measurements of quantum yields can be done using stand-alone integrating sphere setups, which detect all light emitted by the excited molecule and measure the absolute fluorescence quantum yield by comparing the number of emitted photons with the number of absorbed photons.[11]

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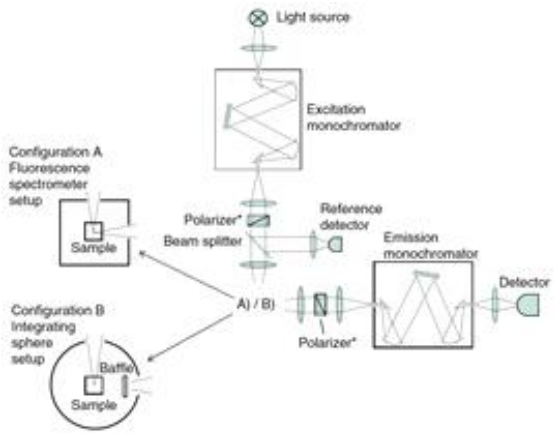

Figure 2. Instrumental setups of a fluorescence spectrometer (configuration A) and of an integrating sphere (configuration B). *Polarizers are not needed for an integrating sphere setup. Source: *Nature Protocols* volume 8, pages 1535-1550 (2013)

1.6. Fluorophore Structure and Properties

Fluorescent compounds can be divided into two categories: intrinsic and extrinsic probes. Intrinsic probes exist in nature and are sufficient to be of practical use. They include aromatic amino acids such as tyrosine and tryptophan, NADH, FAD, FMD, phosphate, chlorophylls, pteridines, among others.[2] Extrinsic probes, on the other hand, can form a covalent or noncovalent complex with the target system. Some examples of extrinsic probes are dansyl chloride, Hoechst dyes, ethidium bromide, iodoacetamide, maleimide and several others. Presently, thousands of fluorescent compounds are synthesized and several of them are commercially available for various applications.

The relationship between molecular structure and fluorescent properties has been recognized and used to synthesize fluorophores. Fluorescence is generally observed in molecules with $\pi \rightarrow \pi^*$ as their lowest energy transition, such as aromatic compounds. Several aliphatic, alicyclic carbonyl and highly conjugated double bond structures exhibit fluorescence as well. In addition, rigidity is another structural feature associated high fluorescence quantum yields. Rigid molecules have less intramolecular thermal motion, which deactivates excited states non-radiatively leading to a reduction in fluorescence intensity.[12] This reduction in fluorescence intensity due non-radiative

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pathways caused by the energy-absorbing flexible group is aptly called the "loose-bolt effect" or "loose-bolt theory".[13]

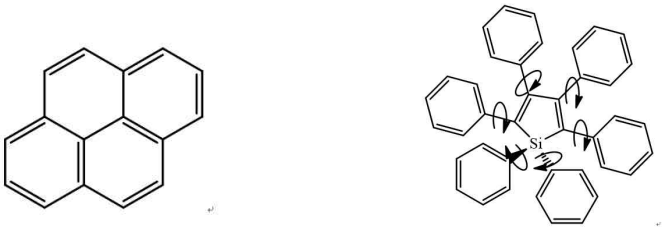

Figure 3. Pyrene (left), an example of a rigid fluorophore and hexaphenylsilole (right), an AIE molecule.

However, this same structural feature has been put to good use with the advent of the aggregation-induced emission (AIE). Due to the extensive conjugation, most fluorophores are hydrophobic and therefore, form non-fluorescent aggregates in aqueous media. Planar luminogens such as pyrene aggregate due to ð-ð stacking interaction. Upon aggregation, the excited states generally decay *via* non-radiative pathways, effectively quenching emission. This behavior is called aggregation-caused quenching (ACQ) of light emission. Several synthetic strategies has been reported to mitigate this problem such as introducing branched chains, bulky groups and rigid scaffolds to act as "spacer" between dye cores.[14,15]

AIE was first observed in a series of silole molecules that were non-luminescent in the solution state but emitted light upon aggregation in 2001.[16] The mechanism behind this unusual phenomenon is not yet completely understood although restriction of intramolecular rotation (RIR), conformational planarization, J-aggregate formation, and twisted intramolecular charge transfer (TICT) were proposed and discussed.[17] Several AIE molecules have rotatable aromatic groups whose molecular motions in solution dissipate the excited-state energy *via* non-radiative pathways, hence without fluorescence. Upon aggregation, the same molecular motions are restricted so de-excitation *via* fluorescence dominates.[18]

2. BODIPY

2.1.Synthesis and General Features

Currently, one of the most extensively studied dye is 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY)due to its sharp

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emission intensity peaks with high fluorescence quantum yields, insensitivity to the pH and polarity of their environment, stability in physiological conditions, non-toxicity and tunability of their photophysical characteristics.[19] The BODIPY core consists of dipyrromethene complexed with a disubstituted boron, most commonly BF_2. BODIPY dyes are typically synthesized from pyrrole with either anhydride, acyl chloride or aldehyde, and from ketopyrroles.

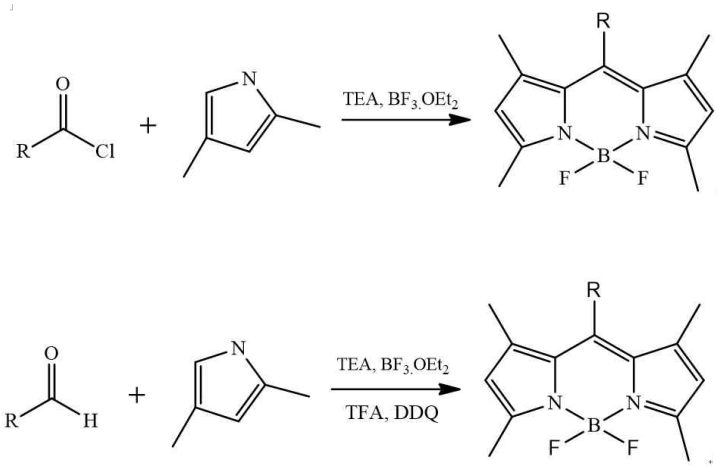

Figure 4. Typical synthetic schemes to afford BODIPY from acyl chloride and pyrrole (top), and from aldehyde and pyrrole (bottom).

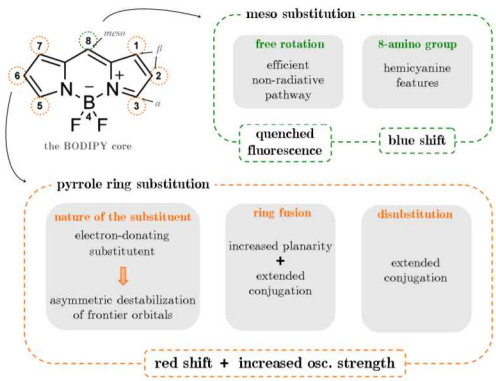

Figure 5. IUPAC numbering of BODIPY core structure and the primary results of modification. Source: Claire Tonnel´e. Chemical Sensors : Modelling the Photophysics of Cation Detection by Organic Dyes. Other. Universit´e Sciences et Technologies - Bordeaux I, 2013. English.[20]

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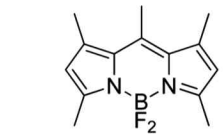
The ease of modification around the core is one of the favorable features of BODIPY. *De novo* approaches where the starting pyrrole already bear the functional groups and post-synthetic derivatization of the dye create a wide-array of BODIPY dyes with diverse characteristics. The meso- or 8-position can be replaced with a nitrogen atom to generate aza-BODIPY. Moreover, positions 1,2,3,5,6,7 in the pyrrole ring are sites of substitution and one or both of the fluorine atoms can be replaced with oxygen to bring about desired changes in the dye.



7

MeOH

λ_{abs} 493 nm, λ_{em} 504 nm

Figure 6. Structure of BODIPY and its corresponding absorbance and emission wavelength. Source: J. Phys. Chem. A 2012, 116, 9621-9631[21]

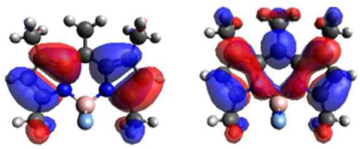


Figure 7. DFT HOMO and LUMO of BODIPY. Source: ACS Appl. Mater. Interfaces 2018, 10, 23254-23262

On a molecular-level, density functional theory (DFT) calculations on the structure of BODIPY reveal that the HOMO and LUMO are characterized as π-orbital. A nodal plane at the meso-position in the HOMO while a large molecular orbital coefficient is found in the same position in the LUMO, suggesting that substitution at the meso-position can tune the LUMO.[22] Consistent with the large LUMO coefficient at the nitrogen atoms, complexation with boron trifluoride mostly affected the LUMO energy. In the HOMO, a nodal plane is found in the pyrrole nitrogen atoms. Aside from these, molecular orbital coefficients are predominantly larger in the HOMO than the LUMO, suggesting that attaching electron donating groups influences the HOMO more than the LUMO of the BODIPY.[23]

These features have spurred the development of BODIPY dyes for bioimaging applications.

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2.2. BODIPY dyes for bioimaging

Over the years, the demand for accurate and efficient detection and visualization of biomolecules have driven the development of fluorescence imaging technique and accordingly, the search for appropriate fluorescent probes. The most widely used and commercially available dyes are coumarin, fluorescein, BODIPY, rhodamine and cyanine dyes.[24,25] However, among them, the relatively new BODIPY has been considered as a potential scaffold for fluorescent bioimaging because it possesses several characteristics ideal as fluorescent probe such as high fluorescence quantum yield, high photostability, non-toxicity and neutral total charge. The structure of BODIPY also allows for diverse modifications to enhance the properties of the dye.

Nonetheless, BODIPY dyes, like other conjugated molecules, are hydrophobic and predisposed to aggregate in subcellular structures. This drawback is circumvented by modifying the dye core with hydrophilic moieties. Conjugating sulfate, phosphate and ammonium ions enhanced the solubility of the dye.[26] However, the presence of charges in these ionic groups may cause unwanted interactions with biomolecules so neutral substituents such as poly(ethylene glycol) chains are also preferred.[27,28]

On the other hand, these bulky chains (PEG) can increase the particle size of the fluorophore and reduce its cell permeability. Solubilizing groups can even account for > 80% of the total molecular weight.[27] Hence, relatively small di/mono-alkoxy and di/mono-acetoxy BODIPYs that exhibit balance between aqueous solubility and lipophilicity to enable cell permeation without random staining of lipid membranes were also explored for cell imaging.[29] Reported di/mono-alkoxy and di/mono-acetoxy BODIPY dyes are accessed by substituting one or two of the fluorine atoms with suitable alcohols. Moreover, results from these researches suggest that substitution of the F atoms has little effect on the photophysical properties (absorption and emission wavelengths) of the dye although electron-donating groups diminish the fluorescence quantum yield while the opposite is observed with electron-withdrawing groups.[30-32]

A newer method of imparting solubility into the BODIPY is by using simple sugars such as lactose, glucose and galactose. In addition, these sugars can also act as targeting moieties of the dye. Lactose has been found to selectively target galectin-1, a beta-galactosidase-binding lectin whose over-expression is linked with breast cancer.[31] On the other hand, glycoconjugation wherein glucose is attached to anticancer agents and drugs is based on the observation that cancerous tissues consume lots of glucose (called Wargburg effect).[32] Lastly, galactose can be used as a broad tumor ligand for targeted cancer therapy.[33] Considering the above

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discussion, the applicability of glucose-conjugated BODIPY dyes has been extended to photodynamic therapy (PDT) and acted on human lung cancer A459 cell line.[34]

Although the modification strategies mentioned previously are effective in imparting aqueous solubility, some BODIPY can be made more biocompatible by being encapsulated within nanocarriers. These structures self-assemble in water and dissociate in the target environment. This transition is accompanied by changes in emission intensity where encapsulated dyes fluoresce weakly due to aggregation but show strong emission once exposed. In this way, the dyes can be made photostable and resistant to photobleaching due to reduced interaction with reactive oxygen molecules.[35-37]

More specific-targeting BODIPY dyes are also made available by using sensitive and selective moieties. Positively charged molecules such as ammonium or phosphonium groups are usually used for mitochondria-targeting fluorescent probes.[40-42] These cations can localize in the negatively-charged mitochondria and can be used to further understand biological events in the organelle.

Cell matrix conditions, such as pH, can also be used to control the localization of the dyes such as BODIPY dyes with weakly basic targeting groups.[43-46] Although cytoplasmic pH is maintained at neutral level, pH levels in endosomes, lysosomes and a few tumor tissues are fairly acidic.

Furthermore, BODIPYs modified with receptor ligands can selectively bind to proteins and hence, visualize the protein of interest.[47-50] BODIPY FL-labeled fluorescent probe can target ERRA (Estrogen-related receptor alpha), an orphan nuclear receptor that is predominantly active in tissues with high metabolic needs and plays an important role in regulating gene expressions used in metabolism.[51]

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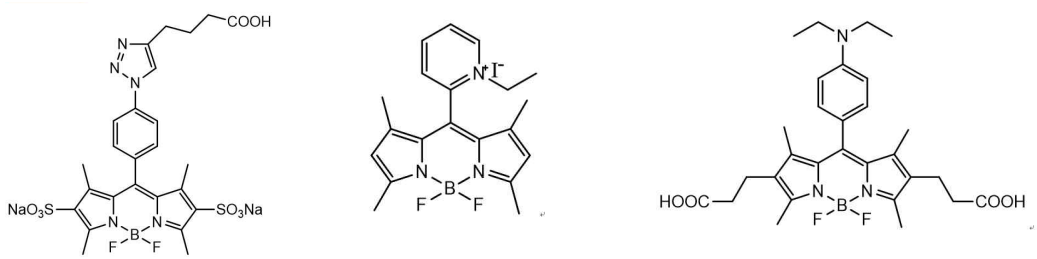

Figure 8. Some representative structures of water-soluble BODIPY (left), BODIPY with a mitochondria-targeting group (middle) and a pH-sensitive BODIPY dye.

2.3. Near-infrared BODIPY dyes

Recent years have seen the development of near-infrared (NIR) emitting fluorescent dyes. The advantages of NIR emitting dyes such as improved sensitivity, high contrast with low background noise, deep tissue penetration and less cell damage prompted the interest for novel NIR fluorophores with enhanced characteristics. [52] Ideally, NIR dyes should absorb and emit within the biological window (650-900 nm), possess high fluorescence quantum yield, narrow excitation/emission, are photostable, have no or negligible toxicity and are biocompatible.[53] Pristine BODIPY, as well as fluorescein, rhodamine, cyanine and Nile red dyes, absorb and emit in the visible spectral region, well below the required wavelength range. Fortunately, NIR dyes are obtained by extending the ð-conjugation within the structure through aromatic ring incorporation, ethynylaryl substitution, aza-substitution at the meso-position and styryl modification.[54]

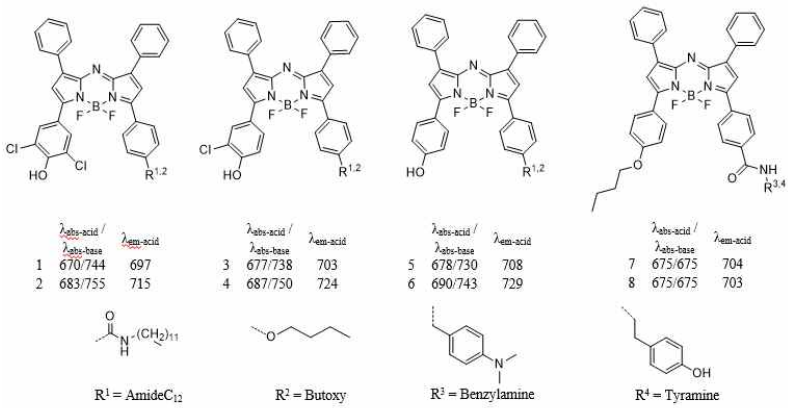

Figure 9. Aza-BODIPY dyes as NIR emitting dyes

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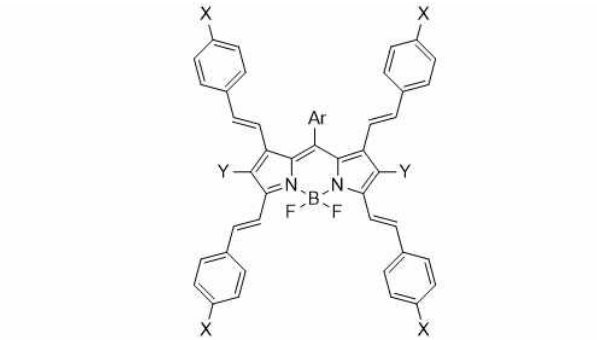

Ar = 3,5-didecyloxyphenyl		λ_{abs} (nm)	λ_{em} (nm)
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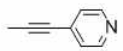
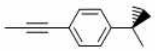
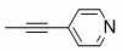
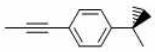
2 = OCH_3	Y = 	721	747
3 = OCH_3	Y = 	732	756
4 = $N(CH_3)_2$	Y = 	802	837
5 = $N(CH_3)_2$	Y = 	797	835

Figure 10. NIR emitting di-styryl modified BODIPY dyes with different substituents.

2.4. Other BODIPY dyes with enhanced photophysical properties

Although great progress has been made in synthesizing NIR-absorbing and selective BODIPY based fluorophores, upon *in vivo* applications, BODIPY dyes are still beset with problems in interacting with the physiological environment. Hence, other BODIPY dyes have emerged with enhanced properties.

2.4.1. Large Stokes shift and BODIPY fluorophores

A common problem with some fluorophores, e.g. fluorescein, rhodamine, oxazine, cyanine, are their smallStokes shifts - generally less than 30 nm.[55] BODIPY is no exception, with commercial dyes BODIPY 493/503 and 505/515having a stokes shift of 10 nm. As explained earlier, Stokes shift is an inherent feature of a specific compound. Small stokes shifts pose a problem with inner-filter effects, poor signal-to-noise ratio and self-quenching due to self-absorption, even at low concentrations. Moreover, fluorophores with large stokes shift are advantageous for exact imaging and sensing.[56] Attaining large stokes-shifted fluorophores

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can be done through a) excited state intramolecular charge transfer (ICT) between donor and acceptor moieties, b) twisted intermolecular charge transfer (TICT) and c) proton transfer, among others. [57-59]

Experimentally, a general strategy to achieve large stokes-shifted fluorophores involving the introduction of vibronic structures has been proposed. This design is based on breaking the symmetric structures of most fluorescent dyes. Although symmetry throughout the HOMO and LUMO endowed fluorophores with high fluorescence quantum yields, this also brought about the weakening of vibrational sub-orbitals. Consequently, excited-state electrons fail to interconvert to lower vibrational states, making the excitation and emission wavelength close to each other.With the introduction of asymmetric structures, vibronic contributions to the HOMO and LUMO can induced efficient internal conversion of the excited-state electrons to lower vibrational states. This then will reduce emission energy and increase the Stokes shift of the fluorophore.[56] For example, 2-(thiophen-2-yl)quinoline appended BODIPY dye with styryl moieties at 1-,3-,5-,7- positions have displayed a large pseudo Stokes' shift of up to 400 nm. This is attributed to the efficient through-bond energy transfer in the nearly planar BODIPY dye.[60] Moreover, a library of asymmetric BODIPY dyes with styryl and carboxylic groups attached to the core exhibited controllable large Stokes' shift ranging from 10 to 51 nm. Steric hindrance and resonance effects have also contributed to the spectral changes.[61]

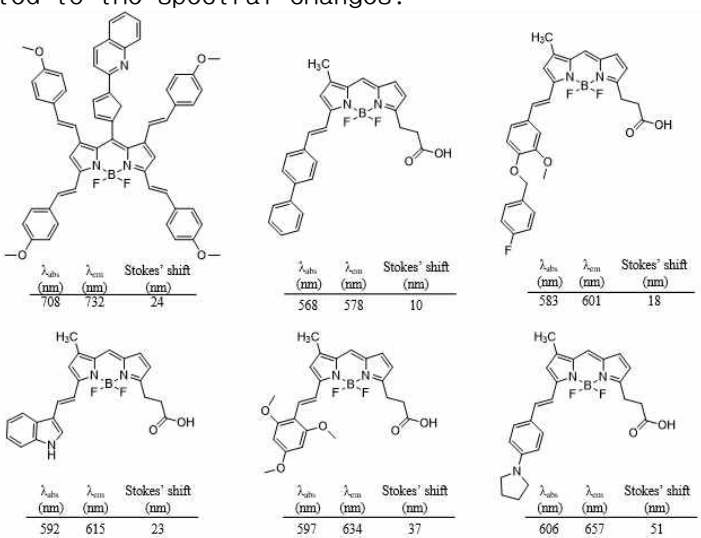

Figure 11. Changes in the Stokes' shift with different substitution patterns.

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CHAPTER 2: Synthesis and Photophysical Characterization of Highly Water-Soluble PEGylated BODIPY Derivatives for Cellular Imaging

1. Introduction

Over the last decade, fluorescence imaging techniques have proved to be powerful tools for visualizing cell biology at many levels and for revealing spatiotemporal details about cellular dynamics.[62-65] They have paved the way for the development of various fluorescent probes, including fluorescent proteins, nanocrystals (quantum dots), and small organic fluorescent dyes, to provide highly sensitive, minimally invasive, and safe detection of cells and tissues.[63,66] However, such fluorescent systems often suffer from several shortcomings which impede their potential application as biological probes. In particular, organic dyes commonly suffer from aggregation-caused fluorescence quenching (ACQ) originating from the formation of non-emissive excimers or energy transfer to quenching sites.[67] To avoid the undesirable quenching effects, bulky protective groups have frequently been introduced to the periphery of the fluorescent core. By this approach, high fluorescence efficiencies can be retained in concentrated solutions, because the sterically bulky protective substituents can prevent intermolecular interactions and unfavorable aggregation.

Among the various organic fluorophores available, 4,4'-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) and its derivatives are widely used due to their sharp fluorescence emissions, large fluorescence quantum yields (QYs ; generally > 0.5), high molar extinction coefficients (typically > 80,000 L $(mol \cdot cm)^{-1}$), modifiability, neutral total charges, and excellent photostability.[36,42,68,69] Although BODIPY and its derivatives have been employed for fluorescence imaging studies, a vast majority of the research concerning the photophysical and electrochemical properties of BODIPY has been carried out in organic environments, whereas counterpart studies conducted in aqueous media are scarce.[70] Various bio-imaging BODIPY dyes have been reported, however they would accumulate in endoplasmic reticulum, mitochondria and subcellular membranes because of their lipophilicity, rigidity and total charge.[68,71-73] Hence, it is of paramount importance to develop BODIPY dyes that are relatively water-soluble under physiological conditions and resist the formation of non-fluorescent aggregates, thereby being suitable for biological and medical use.[39] Appending hydrophilic moieties, such as poly(ethylene glycol) (PEG), *N,N*-bis(2-hydroxyethyl) amine, carbohydrates, nucleotides, or ionic groups like carboxylic acids, sulfonic acids, or

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ammonium groups, to the BODIPY core are some of the approaches recently used to synthesize water-soluble BODIPY dyes.[39,74-78]

Neutral water-soluble BODIPY dyes, such as PEGylated BODIPY, have an advantage over ionic dyes in that they avoid potential electrostatic interactions between the dyes and biomolecules in biological and medical applications.[79] Thus, the use of PEG to increase the water solubility of BODIPY dyes is a technique still widely used among researchers. It is also well known that PEG possesses several biological and medical advantages such as long circulation time, satisfactory biocompatibility, and a tendency to accumulate in tumor sites via the enhanced permeability and retention (EPR) effect of leaky tumor neovasculature.[80] Previous methods for introducing PEG chains as hydrophilic groups to the BODIPY core often involved arylation at the *meso* position of the dye core. This modification would result in an appreciable decrease in the fluorescence QY due to the free rotation of the *meso* substituent, causing energy losses via nonradiative deactivation of excited states.[12,19] In light of the above discussion, we designed a facile synthetic route to prepare highly water-soluble PEGylated BODIPY dyes. Bulky di-branched PEG chains were prepared and introduced at the *meso* position of the BODIPY core, without the incorporation of a rotationally free phenyl ring. We investigated the optical properties and fluorescence quantum yields of the resultant dyes in both organic and aqueous solutions. The BODIPY dyes were well-distributed in the cellular cytoplasm area, wherein they exhibited high fluorescence intensity and low cytotoxicity, and are thus quite promising as bio-imaging agents.

2. Experimental

All reagents were obtained from commercial sources and were used without further purification unless specifically mentioned. 2,4-dimethyl-3-ethylpyrrole, 2,4-dimethylpyrrole, polyethylene glycol monomethyl ether (MPEG, M_n = 350 g mol^{-1}),3,5-dihydroxybenzoic acid and boron trifluoride diethyl etherate were purchased from Sigma-Aldrich Chemical Company (St.Louis, MO, USA). 5-Bromovaleryl chloride was obtained from Tokyo Chemical Industry (Tokyo, Japan). Tetrabutylammonium bromide was obtained from Junsei Chemical Co., Ltd. (Tokyo, Japan). Triethylamine and tosyl chloride were bought from Daejung Chemicals & Metals Co., Ltd. (Siheung, South Korea). Solvents used were of analytical grade and purified using standard techniques. Thin layer chromatography was performed on precoated TLC plates from Merck (Silica Gel 60 F254). NMR Spectroscopic data were obtained from the Korea Basic Science Institute, Gwangju branch. ^1H and ^{13}C NMR spectra were referenced to the residual proton resonance of $CDCl_3$ (δ7.26) and recorded on a JEOL JNM-AL300 spectrometer (Japan) with tetramethylsilane (TMS) as the

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internal standard. ^{11}B (400MHz) NMR spectra were referenced to BF_3Et_2O. ^{19}F (500MHz) NMR chemical shifts were calibrated using CF_3COOH as external standard. Absorption spectra were measured with Hitachi 5300 spectrophotometer from 200 to 900 nm using 10 mm quartz cuvette. Fluorescence emission intensities were measured with Hitachi F-7000 fluorescence spectrophotometer. Absolute fluorescence quantum yields were measured with Hamamatsu Photonics Quantarius - QY Absolute PL quantum yield spectrometer (Hamamatsu Photonics K.K., Hamamatsu, Japan). Cell images were captured using a Zeiss confocal laser scanning microscope. Bruker Ultraflextreme MALDI-TOF/TOF mass spectrometer (Berlin, Germany) at the Ochang Branch of the Korean Basic Science Institute was used to measure the mass of the final compounds.

2.1. Synthesis of halogenated dyes

2.1.1 BODIPY-$(CH_2)_4$-Br

The halogenated dye was prepared according to a previously published method.[81] 5-Bromovaleryl chloride (0.352 ml, 1.00 eq) was added dropwise to a stirred solution of 2,4-dimethylpyrrole (0.500 g, 2.00 eq) in anhydrous dichloromethane at 50° C for 2 h. After the solvent was evaporated, the residual solid was dissolved in toluene (35 ml) and dichloromethane (15 ml). Triethylamine (1.54 ml, 4.20 eq) was added, and the mixture was stirred under argon atmosphere for 30 min, and then boron trifluoride diethyl etherate (1.62 ml, 5.00 eq) was added dropwise. The reaction mixture was refluxed for 1.5 h at 50° C and the solvent was vacuum evaporated. The crude product was purified by silica gel column chromatography eluting with CH_2Cl_2-hexane. Yield49%. ^1H NMR ($CDCl_3$,400MHz): δ = 6.07 (s,2H), 3.46 (t,2H), 2.99 (t,2H), 2.52 (s,6H), 2.43 (s,6H), 2.06 (t,2H), 1.82 (t,2H) ppm.

2.1.2. Ethyl-BODIPY-$(CH_2)_4$-Br

This compound was prepared in the same way as **BODIPY-$(CH_2)_4$-Br**, using 2,4-dimethyl-3-ethylpyrrole (0.500 g, 2.00 eq). All other reagents were scaled accordingly. The crude product was purified by silica gel column chromatography eluting with CH_2Cl_2-hexane. Yield 56%. ^1H NMR ($CDCl_3$, 400MHz): δ = 3.46 (t,2H), 3.03 (t,2H), 2.50 (s,6H), 2.41 (m,4H), 2.35 (s,6H), 2.07 (t,2H), 1.83 (t,2H), 1.05 (t,6H) ppm.

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2.2 Synthesis of Poly(ethyleneglycol)methyl ether tosylate

The PEG moiety was synthesized according to the previously reported procedure.[82] Briefly, 350 MW monomethyl polyethylene glycol (5.00 g, 1.00 eq) was dissolved in 5.00 mL of anhydrous pyridine. Tosyl chloride (3.27 g, 1.20 eq) was dissolved in anhydrous methylene chloride and slowly injected into the solution. The solution was continuously stirred under Ar gas for 8 h at RT. Then, 6N HCl solution (5 times the total volume of pyridine used) was added into the solution. The mixture was extracted with dichloromethane three (3) times and the organic layer was washed with 2N HCl solution. After drying with $MgSO_4$,thesolvent was evaporated to afford the tosylated PEG as a colorless oil. Yield 76%. ^{1}H NMR ($CDCl_3$, 400MHz) : δ=7.81 (d,2H), 7.36 (d,2H), 4.16 (t,2H), 3.68-3.61 (m,32H), 3.58 (t,2H), 3.38 (s,3H), 2.45 (s,3H) ppm.

2.3. Synthesis of Ethyl-3,5-dialkoxy benzoate

Ethyl-3,5-dihydroxy benzoate (0.636 g, 1.00 eq), PEG methyl ether tosylate (4.00 g, 2.2 eq) and anhydrous K_2CO_3 (2.12g,4.4eq) were dissolved in anhydrous DMF and the solution was refluxed for 12 h at 80° C under Ar atmosphere.[82] Then, the solution was poured into ice-cold water and extracted with dichloromethane three (3) times. The organic layer was washed with water and brine, and dried over $MgSO_4$,following which the solvent was removed by rotary evaporation. The crude product was purified by silica gel column chromatography using CH_2Cl_2 and MeOH as eluent. Yield53%.^{1}H NMR ($CDCl_3$, 400MHz): δ= 7.20 (s,2H), 6.69 (s,1H), 4.37 (m,2H), 4.16 (t,4H), 3.76-3.60 (m,52H), 3.54 (t,4H), 3.38 (s,6H), 1.38 (t,3H) ppm.

2.4. Synthesis of 3,5-dialkoxy benzoic acid

In a THF-H_2O (1:1) mixed solvent system, ethyl-3,5-dialkoxy benzoate (3.47g,1.00eq) and sodium hydroxide (0.473g, 3.00 eq) were dissolved and refluxed for 24 h at 80° C. After the solution cools to room temperature, the pH was adjusted to 3-4 using 10% v/v HCl. Then it was extracted using CH_2Cl_2, dried over $MgSO_4$ and concentrated *via* rotary evaporation to afford a brown oil. Yield 13%. ^{1}H NMR ($CDCl_3$, 400MHz): δ= 7.25 (s,2H), 6.70 (s,2H), 4.16 (t,4H), 3.71-3.65 (m,52H), 3.57 (m,4H), 3.38 (s,6H) ppm.

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2.5. Synthesis of PEGylated BODIPY

To KOH (5.00 mg, 1.00 eq) dissolved in MeOH, 3,5-dialkoxy benzoic acid (150 mg, 2.00 eq) was added and stirred. Then the solvent was completely removed to obtain potassium 3,5-dialkoxy benzoate. BODIPY-$(CH_2)_4$-Br (43.0 mg, 20 eq), potassium 3,5-dialkoxybenzoate (200 mg, 40 eq) and tetrabutylammonium bromide (1.81 mg, 1 eq) were dissolved in anhydrous DMF and refluxed at 40° C for 48h. After cooling to room temperature, the solvent was vacuum distilled, and the crude product was purified by silica gel column chromatography eluting with CH_2Cl_2: MeOH to afford **BOD-PEG** dye as a dark orange oil. Yield 17%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.16 (d-s,2H), 6.69 (s,1H), 6.06 (s,2H), 4.36 (t,2H), 4.12 (t,4H), 3.69-3.64 (m,52H), 3.54 (t,4H), 3.38 (s,6H), 3.03 (m,2H), 2.51 (s,6H), 2.41 (s,6H), 1.96 (m,2H), 1.80 (m,2H) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz): δ 186.30, 177.96, 161.47, 160.01, 156.01, 135.43, 132.00, 130.11, 121.96, 108.13, 106.93, 101.70, 92.41, 72.02, 71.45, 70.92, 70.75, 70.66, 69.67, 67.78, 59.13, 42.78, 32.20, 29.74, 27.90, 16.39, 14.49. ^{19}F NMR ($CDCl_3$, 500 MHz): δ = -147.10 (m) ppm. ^{11}B NMR ($CDCl_3$, 400 MHz): δ = 0.410 (t) ppm. MALDI-TOF/TOFMS: $[M+Na]^+$ m/z: calculated for $C_{58}H_{95}BF_2N_2NaO_{20}$, 1211.64; found, 1213.713.

EtBOD-PEG was synthesized using the same procedure described above but with ethyl-BODIPY-$(CH_2)_4$-Br (40.0 mg, 1.00 eq). All the other reagents were scaled accordingly, and the crude product was purified by silica gel column chromatography eluting with CH_2Cl_2: MeOH to afford the PEGylated BODIPY as a dark magenta oil. Yield 25%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.17 (d-s,2H), 6.70 (s,1H), 4.37 (t,2H), 4.12 (t,4H), 3.71-3.63 (m,52H), 3.56 (t,4H), 3.38 (s,6H), 3.07 (m,2H), 2.49 (s,6H), 2.40 (m,2H), 2.33 (s,6H), 2.05 (m,2H), 1.99 (t,2H), 1.03 (t,6H) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz): δ 187.42, 166.45, 160.02, 152.55, 144.06, 138.63, 135.75, 132.91, 132.06, 131.05, 108.18, 106.82, 100.06, 72.03, 71.46, 70.92, 70.76, 69.67, 67.78, 59.14, 42.79, 29.75, 29.18, 17.19, 16.09, 14.86, 13.37, 12.43. ^{19}F NMR ($CDCl_3$, 500 MHz): δ = -146.43 (m) ppm. ^{11}B NMR ($CDCl_3$,400MHz): δ = 0.458 (t) ppm. MALDI-TOF /TOFMS: $[M+Na]^+$ m/z: calculated for $C_{62}H_{103}BF_2N_2NaO_{20}$, 1267.71; found,1267.786.

2.6. Cell proliferation assay

The human breast MCF-7 cells (3 × 10^3cells/well) were seeded in 96-well plates. After the cells were maintained for 24 h, cells were treated with either **BOD-PEG** or **EtBOD-PEG** dye for 24 h. Cell proliferation assay was measured using CellTiter 96 ® AQueous One

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Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instruction. This assay contains MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] which is reduced to formazan by the action of NADH or NADPH in metabolically active cells.[83] The absorbance value of the wells containing solutions of MTS (background) was subtracted from those of the wells containing the treated and control cells.

2.7. Confocal microscopy

MCF-7 cells were grown on sterilized coverslips in 12-well overnight and then treated with either **BOD-PEG** or **EtBOD-PEG** dye for 24 h. After washing with phosphate-buffered saline (PBS), cells were fixed with 4% paraformaldehyde for 60 min and permeabilized with 0.5% Triton X-100 for 10 mins at room temperature. The slides were mounted with Vectashield mounting medium with 4′,6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions. Images were captured with a confocal microscope at 20× magnification (LSM-700; Carl Zeiss Microimaging Inc., Stockholm, Sweden).

3. Results and Discussion

3.1. Synthesis of PEGylated BODIPY Dyes

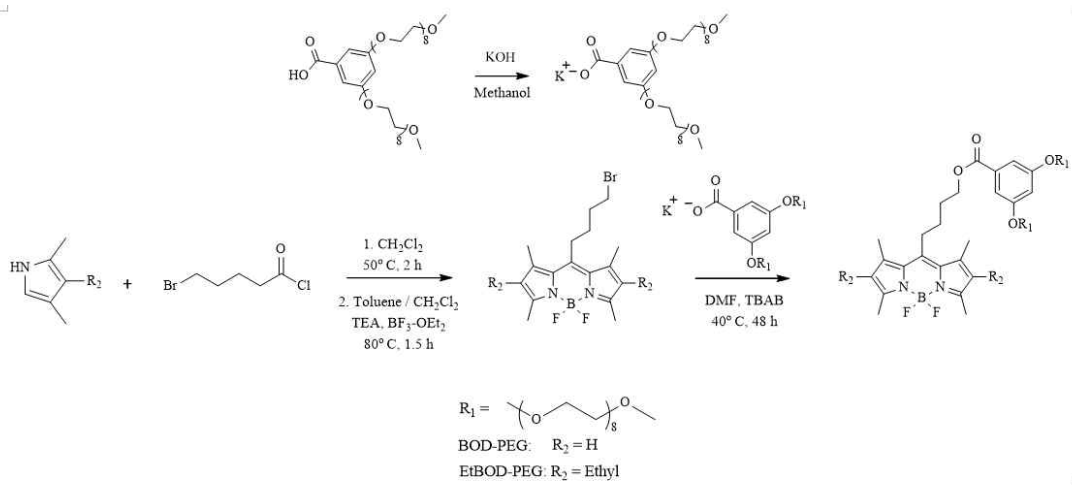

Figure 12. Synthesis of PEG-functionalized BODIPY fluorescent dyes BOD-PEG and EtBOD-PEG.

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Figure 12 outlines the synthesis of the fluorescent BODIPY dyes containing di-branched PEG chains. The di-branched PEG chains were prepared according to previously reported methods.[82] First, Williamson etherification between ethyl 3,5-dihydroxybenzoate and tosylated PEG produced di-PEGylated benzoates. Their subsequent hydrolysis yielded di-PEGylated benzoic acid. *Meso*-1-bromo-butyl-substituted BODIPY dyes were prepared via the condensation of 5-bromovaleryl chloride with either 2,4-dimethyl-3-ethylpyrrole or 2,4-dimethylpyrrole and subsequent complexation with $BF_3 \cdot OEt_2$ in the presence of triethylamine. Finally, the esterification between the bromine-containing BODIPYs and the carboxyl ends of the di-PEGylated benzoic acid afforded the water-soluble BODIPY dyes in moderate yields; 16.6% and 25.2% for **BOD-PEG** and **EtBOD-PEG**, respectively. The formation of an ester bond is evidenced by the appearance of triplet peaks at 4.36/4.37 ppm in the ^1H NMR spectra. All intermediate compounds were characterized by ^1H NMR, and the final products were also characterized by ^{13}C NMR, ^{19}F NMR and ^{11}B NMR. High-resolution masss pectral analysis showed the PEG chains to be approximately 6-8 units in length.

3.2. Photophysical properties

Dye	Solvent	λ_{abs}/nm	λ_{em}/nm	QY[c]
BOD-PEG[a]	THF	498	516	0.681
	CH_2Cl_2	500	517	0.663
	EtOH	498	515	0.668
	H_2O	496	511	0.512
EtBOD-PEG[b]	THF	522	541	0.659
	CH_2Cl_2	524	541	0.658
	EtOH	522	539	0.624
	H_2O	520	535	0.254

Table 1. Photophysical properties of BOD-PEG and EtBOD-PEG dyes in different solvents at room temperature (concentration = 17.2 μM). [a]For ϕ_{fl} measurements, λ_{ex}= 500 nm. [b]For ϕ_{fl} measurements, λ_{ex}= 520 nm. [c]QY= Absolute fluorescence quantum yield.

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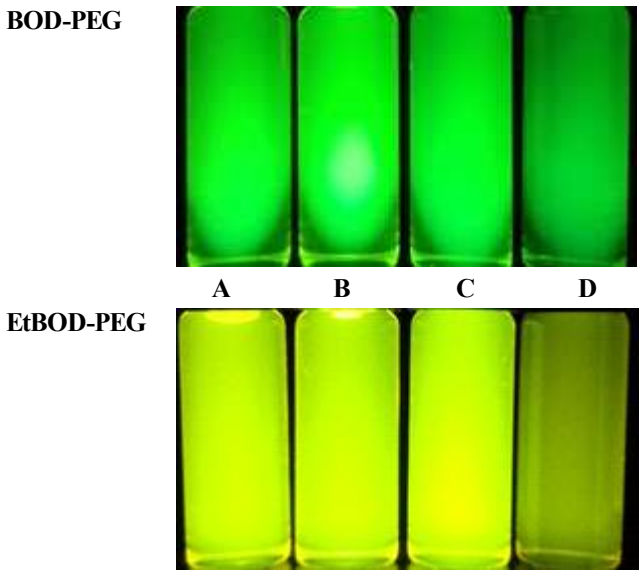

Picture 1. Photographs of BOD-PEG and EtBOD-PEG under UV illumination (365 nm) in different solvents: (A) THF; (B) CH_2Cl_2; (C) EtOH; (D) H_2O (concentration = 17.2 μM).

The photophysical properties of **BOD-PEG** and **EtBOD-PEG** were investigated in different solvents, and are summarized in **Table 1**. As depicted in **Figures 13** and **14**, narrow absorption spectra mirroring the respective emission spectra were recorded for both fluorescent dyes. The absorption and emission profiles correspond to those of typical BODIPY derivatives,[84] indicating that the appended PEG did not exert a significant effect on the photophysical properties of the dye in any of the solvents used in this study. The absorption spectra of **BOD-PEG** exhibited maximum intensity (λ_{ab}) at 498-500 nm in organic solvents and at 496 nm in water, due to the π-π* transition of the BODIPY unit. The λ_{ab} generally blue-shifts with an increase in the polarity of the solvent, but only by a few nanometers. The emission peaks of **BOD-PEG** were observed at 515-517 nm in organic solvents and at 511 nm in water. The presence of ethyl groups at the 2,6 positions of the BODIPY core in the dye **EtBOD-PEG** resulted in red-shifts in the absorption spectra of ~20 nm relative to its unsubstituted counterpart (520-524 nm), to 539-541 nm in organic solvents and 535 nm in water. Theoretical studies have indicated that electron-withdrawing substituents stabilize the lowest unoccupied molecular orbital (LUMO) of BODIPY units, while electron-donating substituents destabilize the LUMO.[85,86] Therefore, electron-donating ethyl

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units resulted in the red-shifted emission of **EtBOD-PEG** relative to that of **BOD-PEG**.

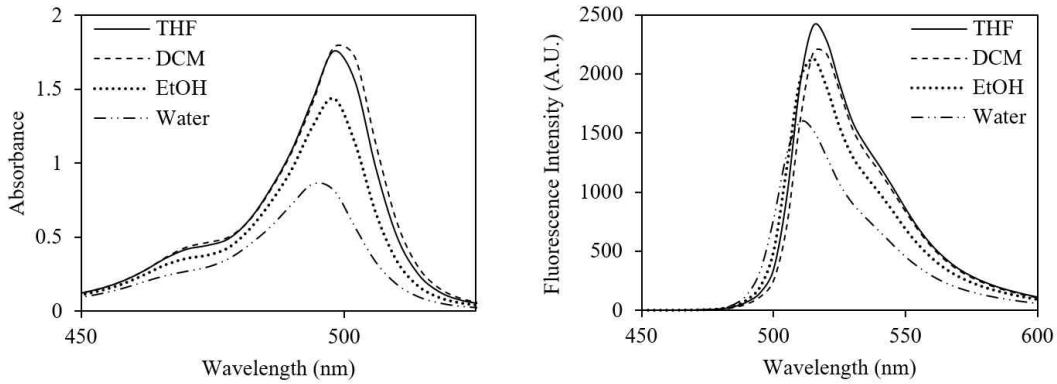

Figure 13. Absorption (left) and emission (right) spectra (λ_{ex}= 496-500 nm) of BOD-PEG dye in different solvents (concentration = 17.2 μM).

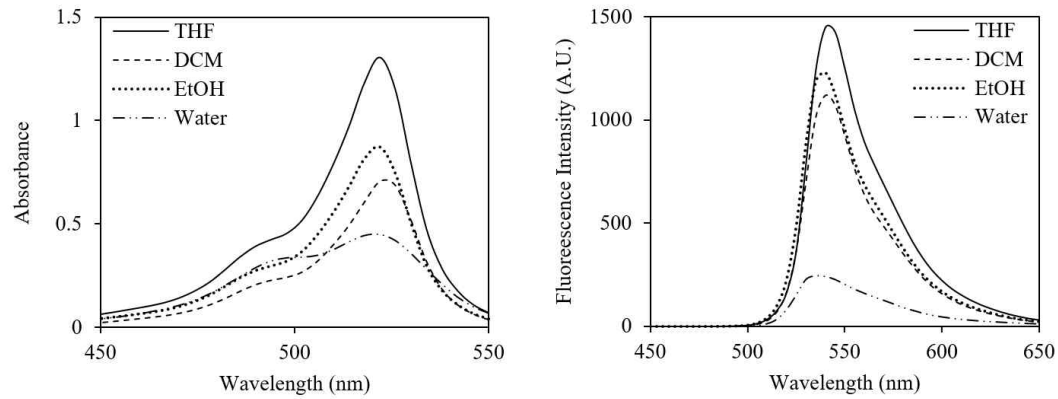

Figure 14. Absorption (left) and emission (right) spectra (λ_{ex}= 520-524 nm) of **EtBOD-PEG** dye in different solvents (concentration = 17.2 μM).

In the case of EtBOD-PEG, broadening of the peak at 480-500 nm in the absorption profile in water seemed to suggest dye aggregation. To understand this behavior further, we studied the fluorescence spectra and fluorescence QY of both dyes in aqueous solution as a function of concentration, as depicted in **Figure 15**. The fluorescence spectra of BODIPY dyes were red-shifted by about 30 nm and the emission intensities decreased exponentially as the dye concentrations were increased from 1.0 μM to 10.0 μM. This phenomenon may be ascribed to intermolecular interactions or aggregation in aqueous solution induced by the

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increasing concentration of the dyes.[87] The QYs of the dyes in water at 1.0 μM reached 0.514 for **BOD-PEG** and 0.471 for **EtBOD-PEG**. At concentrations of 30.0 μM, the fluorescence QYs decreased to 0.361 for **BOD-PEG** and 0.138 for **EtBOD-PEG** (**Table 2**). Notably, both dyes exhibited relatively high fluorescence QYs even at concentrations of 30.0 μM, because the aggregation of the dyes in water was suppressed by the hydrophilic and sterically hindering groups. Further, relative to the unsubstituted **BOD-PEG**, the ethyl groups at the 2- and 6-positions in **EtBOD-PEG** increased the hydrophobic character of the dye core. In a study conducted by Nepomnyashchii *et al.*, 2,6-ethyl-substituted BODIPY dyes displayed lower fluorescence than their unsubstituted counterparts,[70] suggesting that hydrophobic interactions between the ethyl-substituted BODIPY dyes may play a role in this phenomenon.

Dye	Conc. (μM)	ϕ_{fl}
BOD-PEG[a]	1.0	0.514
	10.0	0.561
	30.0	0.361
EtBOD-PEG[b]	1.0	0.471
	10.0	0.292
	30.0	0.138

Table 2. Absolute fluorescence quantum yields of **BOD-PEG** and **EtBOD-PEG** dyes in water at different concentrations.
[a] λ_{ex}= 500 nm. [b] λ_{ex} = 520 nm.

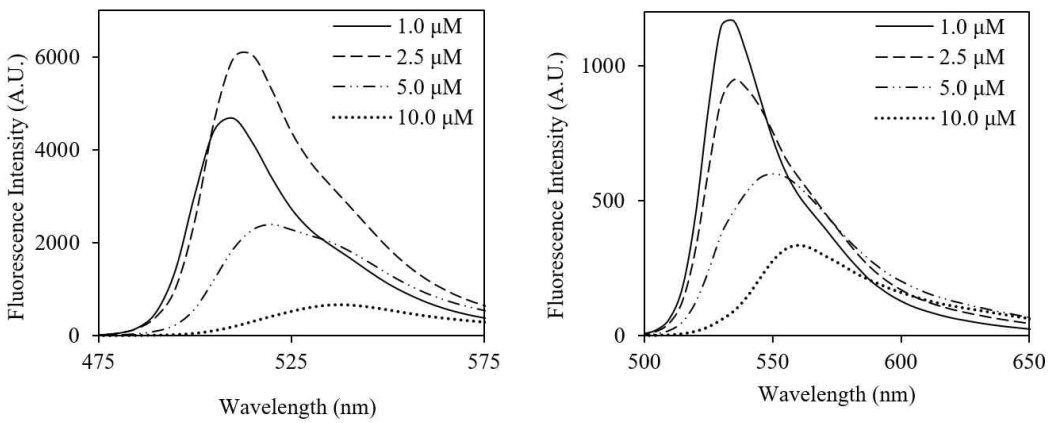

Figure 15. Emission spectra of BOD-PEG (left, λ_{ex}= 506 nm) and EtBOD-PEG (right, λ_{ex}= 530 nm) dyes in water at different concentrations.

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3.3 Cell viability and imaging

One of the critical requirements for fluorescent probes is good biocompatibility.[42] The CellTiter 96 ® AQueous One Solution Cell Proliferation Assay was used to assess the cytotoxicity of the BODIPY dyes. This method is based on the bioreduction of the tetrazolium compound MTS (Owen's reagent) to a colored formazan product by the cells. The quantity of formazan produced, as measured by absorbance at 490 nm, is directly proportional to the number of viable cells.[83] Cell viability experiments for the BODIPY dyes synthesized in this study were carried out employing MCF-7 human breast cancer cells. The test cells were incubated with either **BOD-PEG** or **EtBOD-PEG** dye at concentrations ranging from 2.5 to 10 μM. As shown in **Fig. 16**, there was no significant reduction in the viability of the cells after 24 h incubation with the fluorescent dyes. The BODIPY probes did not exhibit signs of toxicity towards the tested cells at low concentrations. Moreover, it is noteworthy that the number of living cells was maintained at approximately 90% and 80% of the original count for **BOD-PEG** and **EtBOD-PEG**, respectively, even after treatment with 10 μM of the dye. These results indicate that neither dye is significantly cytotoxic under the tested conditions over 24 h.

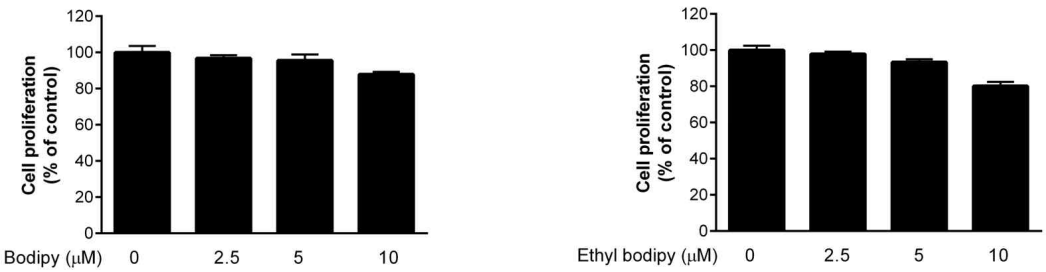

Figure 16. The relative number of viable cells after incubation with different concentrations of **BOD-PEG** (top) and **EtBOD-PEG** (bottom) dyes for 24 h.

To evaluate the potential application of the fluorescent dyes for bioimaging, MCF-7 cells were treated with the dyes for 24 h and imaged by confocal microscopy. 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent stain commonly used to visualize the nucleus, is employed to confirm the localization of the fluorescent dyes within the cells.[88] As shown in **Figure 17**, there was no fluorescence emission from the control untreated cells, while the test cells incubated with the dyes at concentrations of 10 μM emitted bright green fluorescence of high intensity. Additionally, the fluorescence microscopy images of the MCF-7

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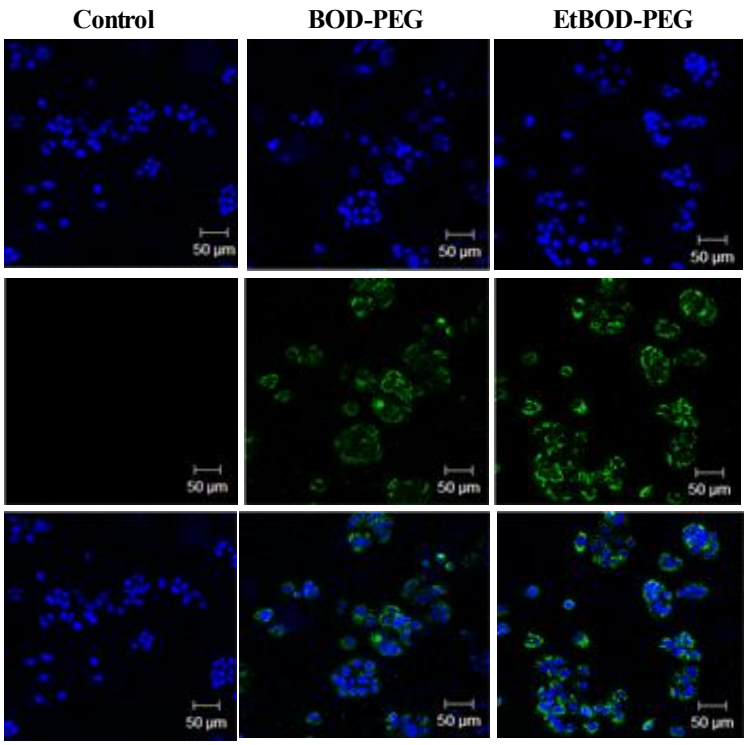
cells with the fluorescent probes showed that BODIPY dyes were successfully taken in by the cells, efficiently localized, and then fluoresced upon excitation.



Picture 2. CLSM images of MCF-7 human breast cancer cells with either BOD-PEG or EtBOD-PEG dye at various concentrations, with the nucleus labeled with 4′ ,6-diamidino-2-phenylindole (DAPI). Top: DAPI images; middle: BOD-PEG/ EtBOD-PEG dye images; bottom: merged images of both (concentration = 10.0 μM).

4. Conclusion

In this study, a series of water-soluble PEGylated BODIPY dyes (BOD-PEG and EtBOD-PEG) were prepared, and their photophysical properties were investigated. Bulky di-branched PEG chains were introduced at the *meso* position of the BODIPY core to reduce the aggregation tendencies of the dyes. The dye BOD-PEG, which has no substitutions at positions 2 and 6 of the BODIPY core, exhibited absorption and emission maxima at shorter wavelengths relative to those of EtBOD-PEG, which has electron-donating ethyl groups at the 2 and 6 positions of the core. Notably, the

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fluorescence QYs of the dyes at 1 μM in water were 0.514 for BOD-PEG and 0.471 for EtBOD-PEG, which are higher than those of other BODIPY-based water-soluble probes reported previously. The PEGylated BODIPY dyes were able to permeate MCF-7 cells and localized in the cellular cytoplasm, exhibiting good water solubility and biocompatibility. This work may provide new strategies for the design and fabrication of highly efficient fluorescent probes in aqueous environments.

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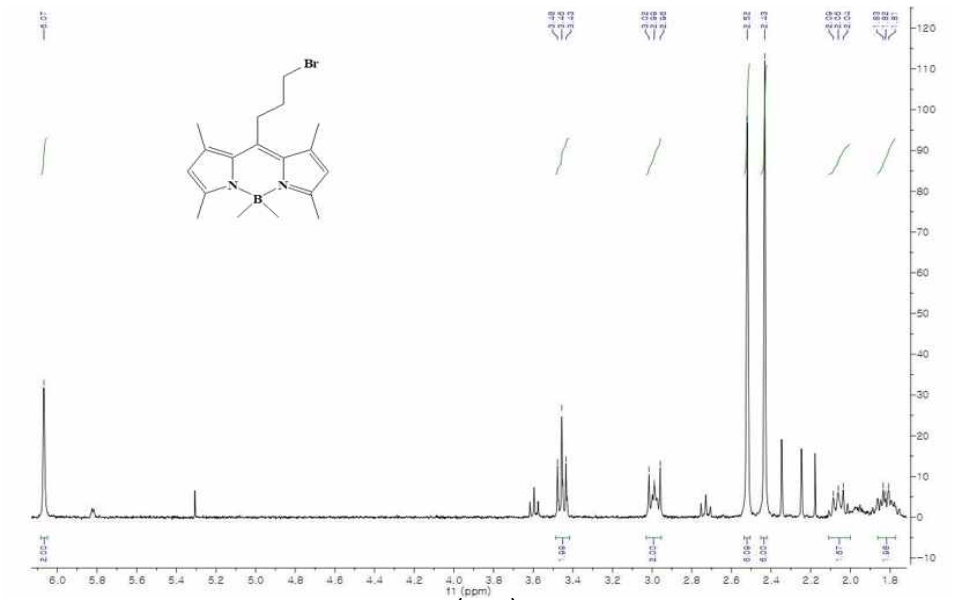
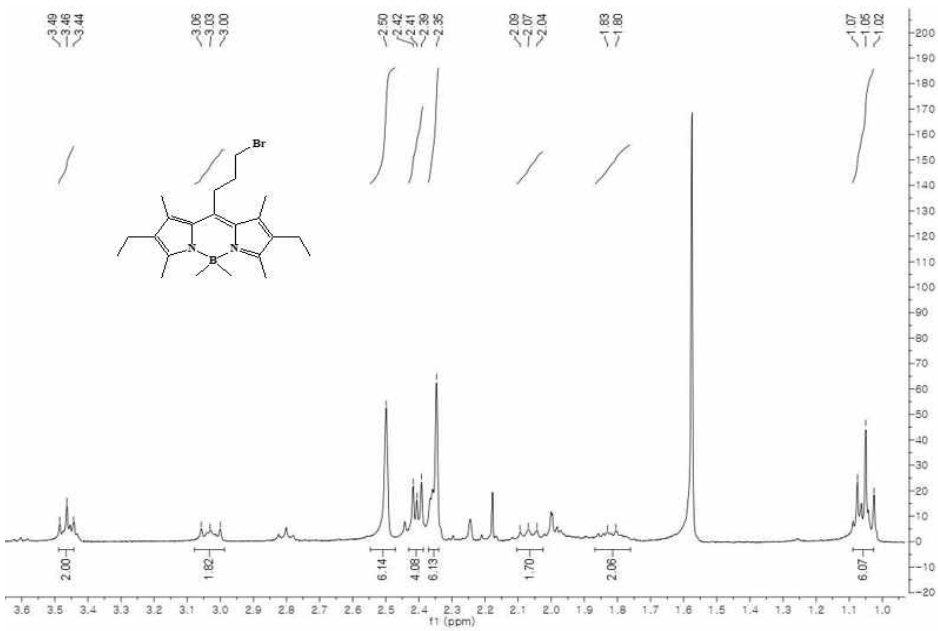


BODIPY-$(CH_2)_4$-Br



Ethyl-BODIPY-$(CH_2)_4$-Br

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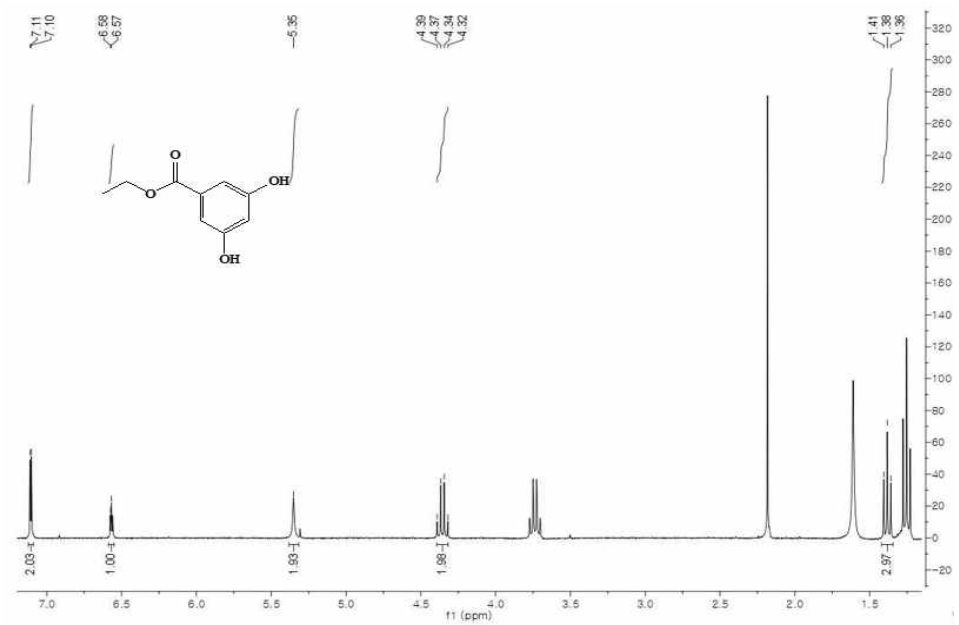
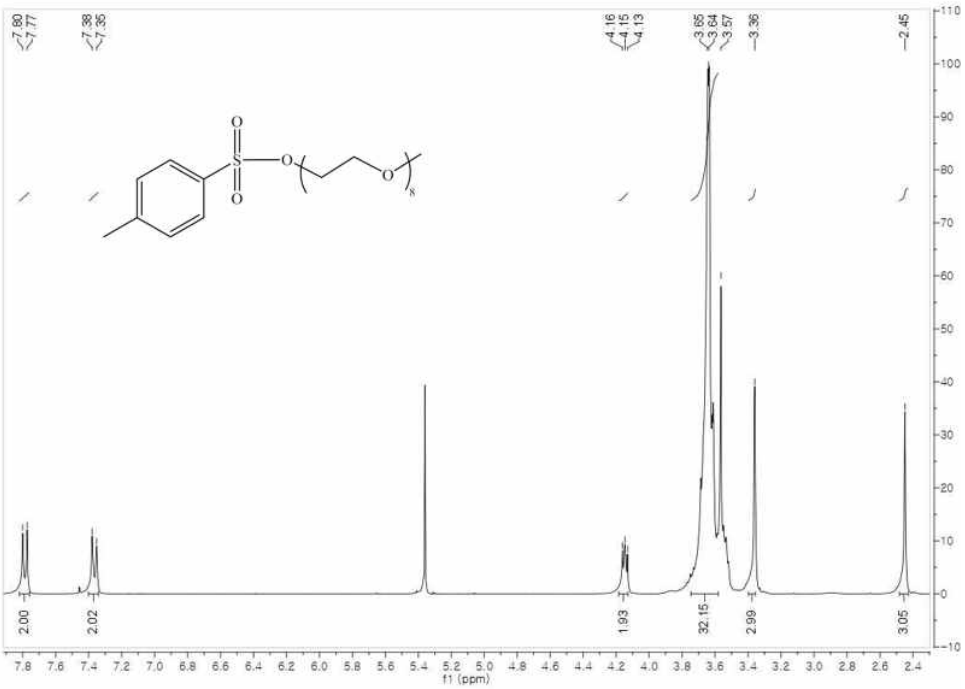


Ethyl-3,5-dihydroxy benzoate



Poly(ethylene glycol) methyl ether tosylate

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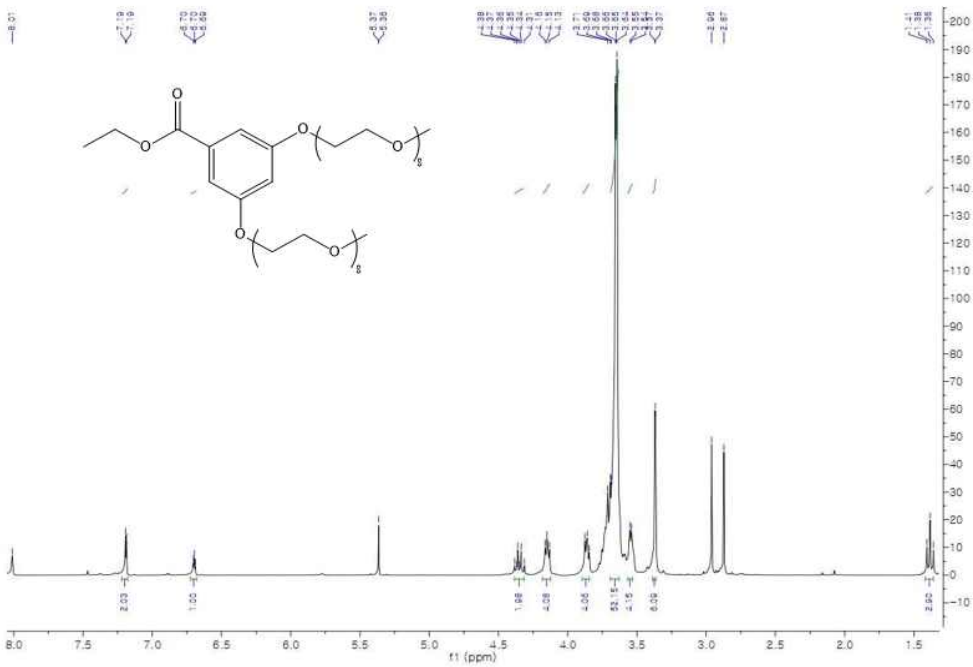
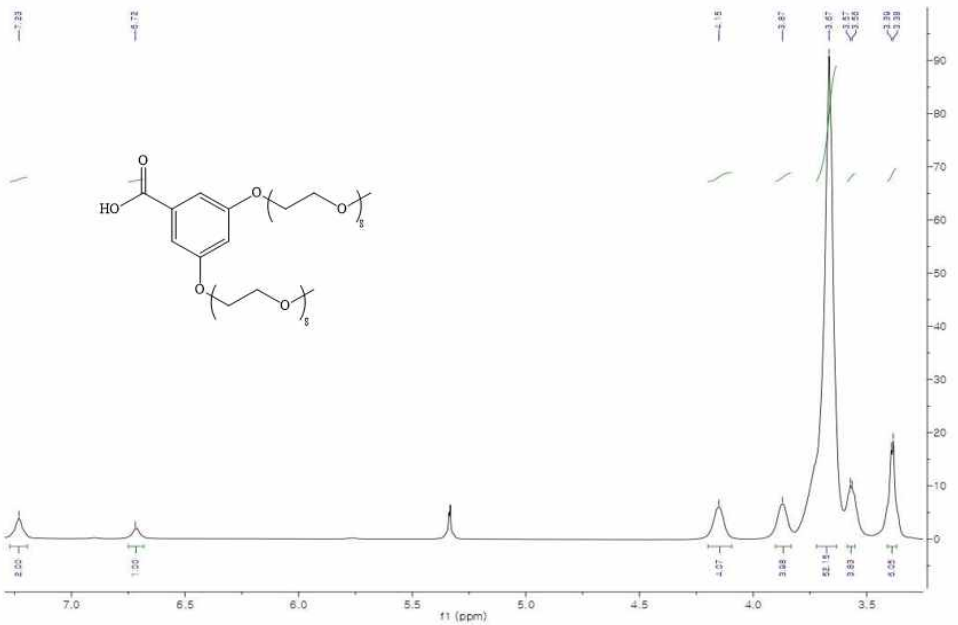


Ethyl-3,5-dialkoxy benzoate



3,5-dialkoxy benzoic acid

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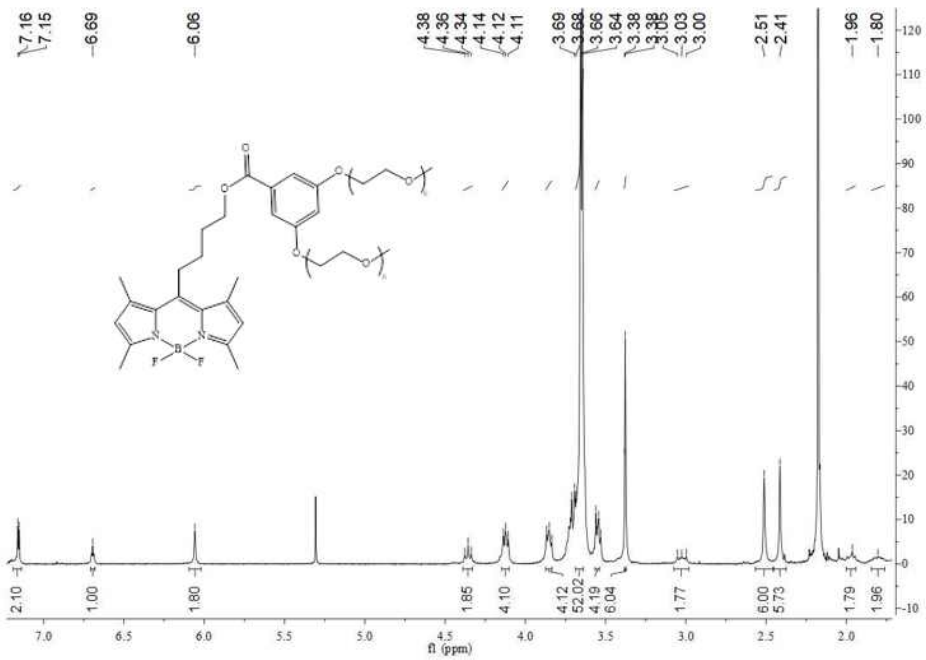
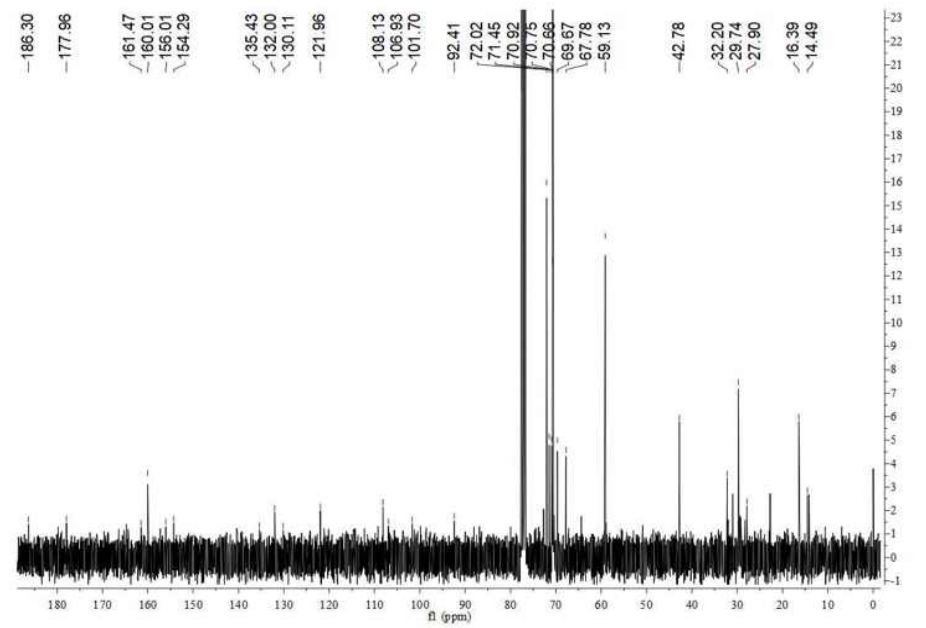


BOD-PEG



BOD-PEG-^{13}C NMR

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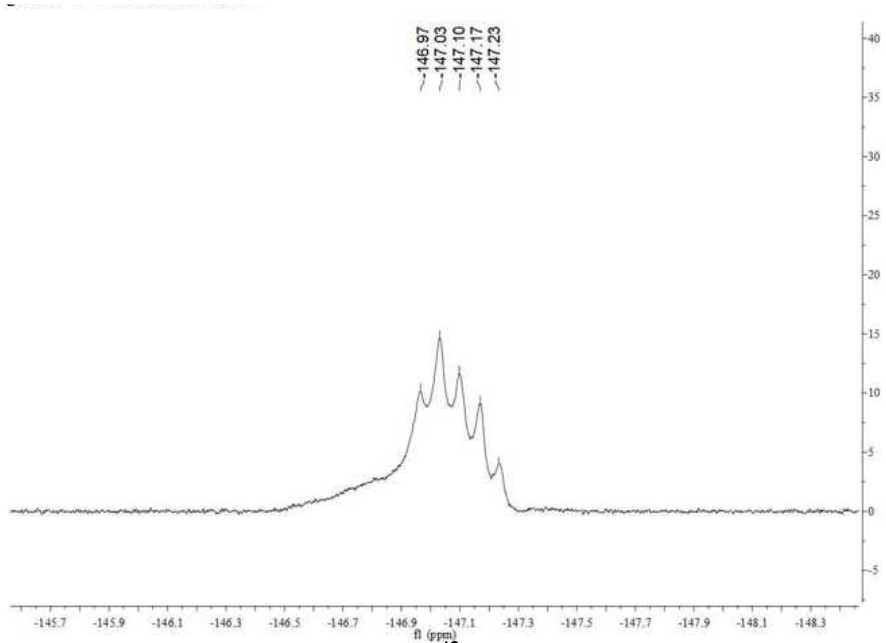
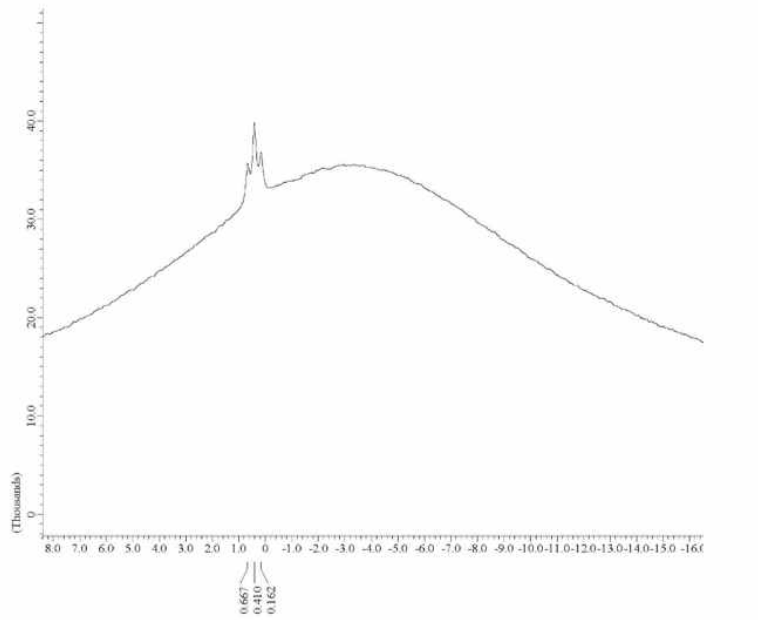


BOD-PEG ^{19}F NMR



BOD-PEG ^{11}B NMR

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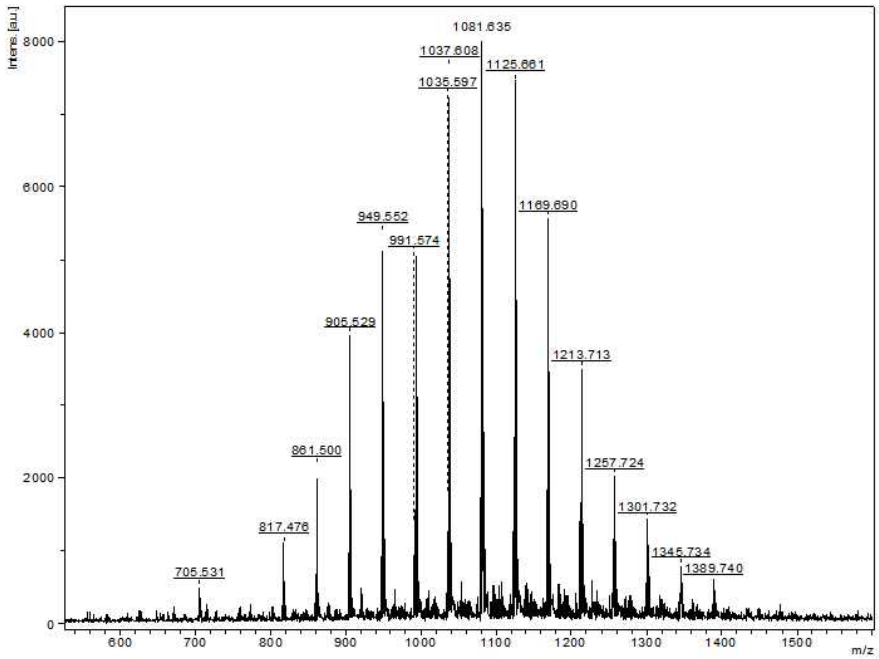
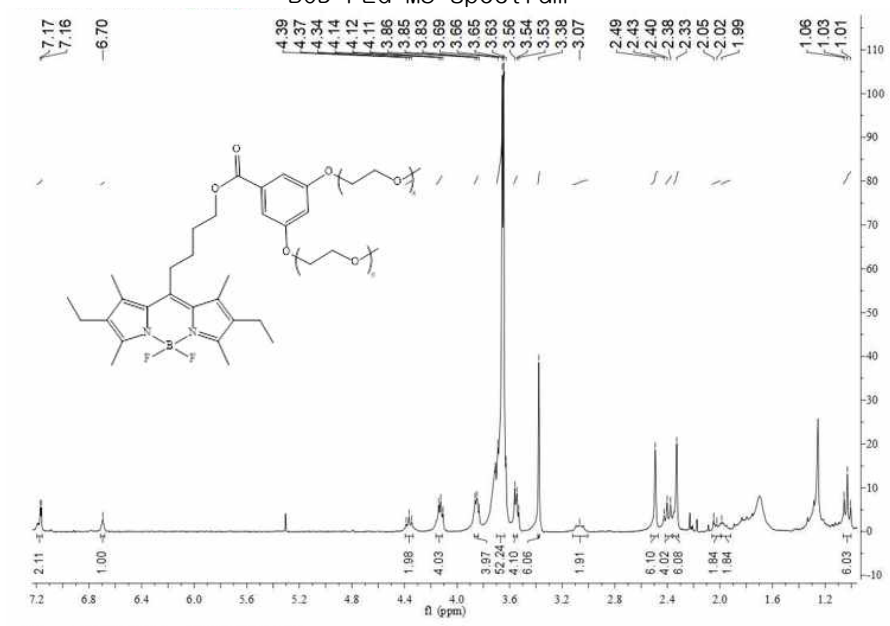


BOD-PEG MS spectrum



EtBOD-PEG

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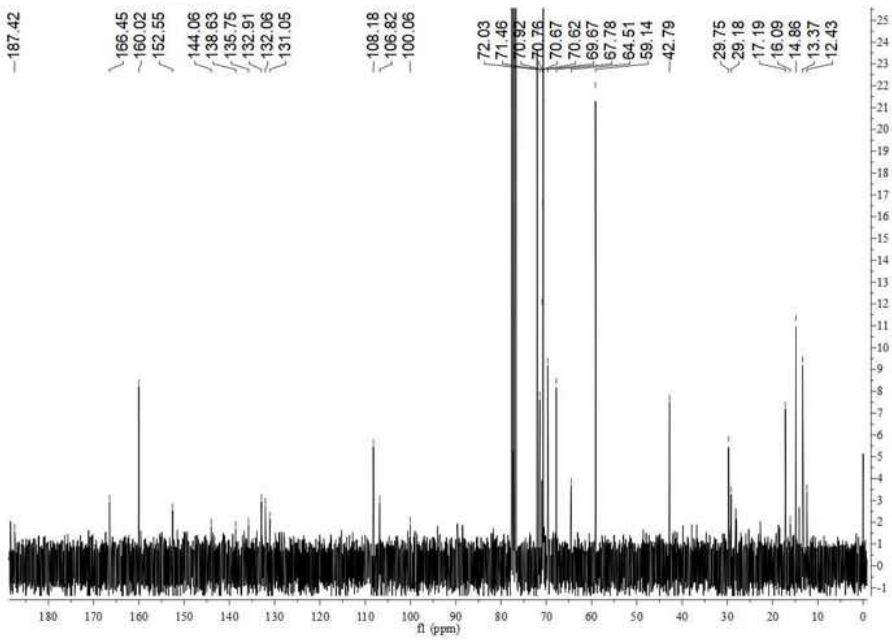
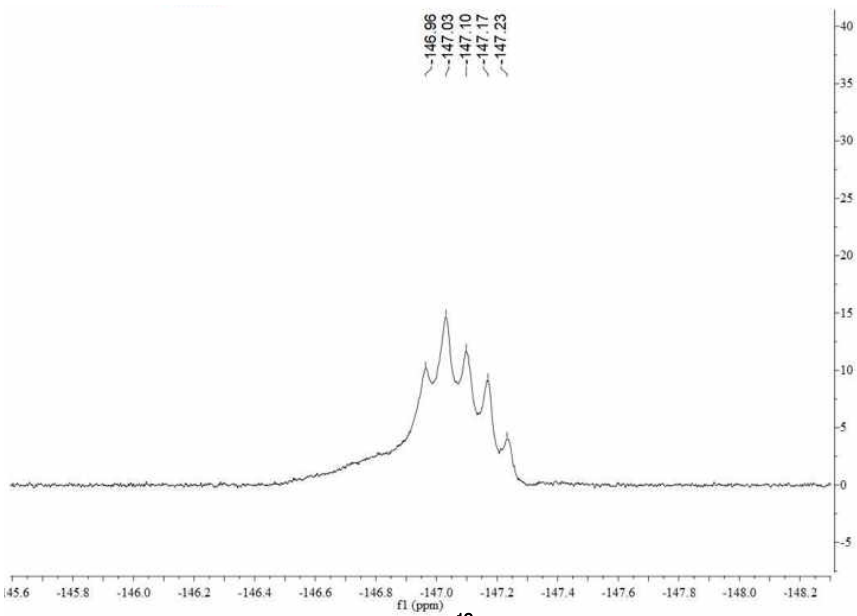


EtBOD-PEG ^{13}C NMR



EtBOD-PEG ^{19}F NMR

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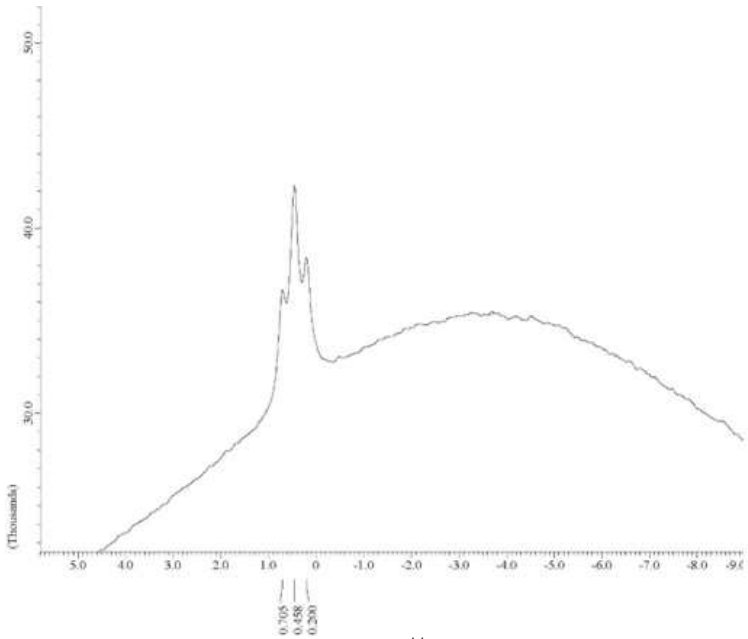
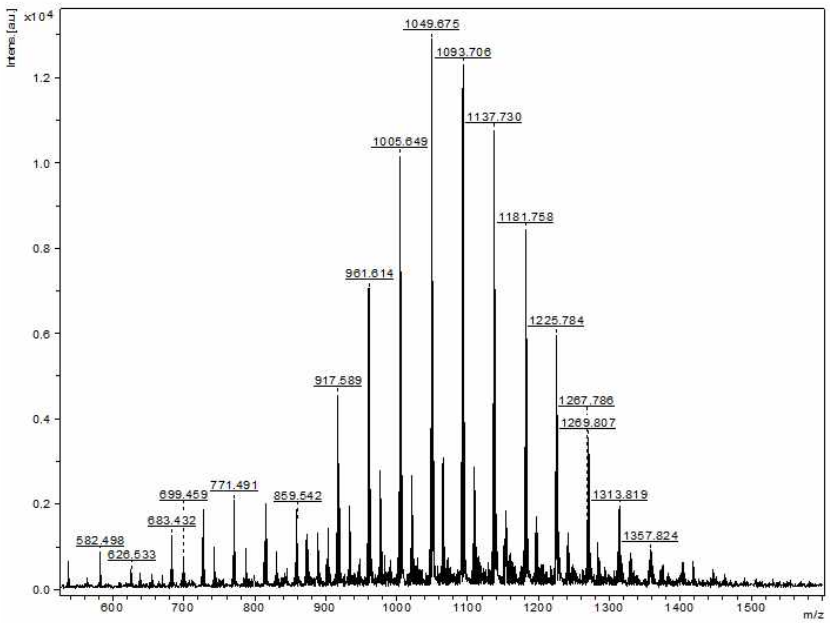


EtBOD-PEG ^{11}B NMR



EtBOD-PEG MS spectrum

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CHAPTER 3: Multi-Responsive Hydrogels Functionalized with a Photochromic Spiropyran-Conjugated Chitosan Network

1. Introduction

The development of photochromic hydrogels has been of great interest because of their potential application in optical memory devices, drug delivery platforms, and chemical sensors.[89-93] Phenoxyquinone, spiropyran, bisthienylethene, azobenzene, and other compounds are typically employed as photo-responsive building blocks that undergo various types of structural isomerization processes such as phenyl group migration, ring opening, ring closure, and cis-trans isomerization under specific light irradiation.[94-96] Of these compounds, spiropyran derivatives have garnered great attention because of their striking color changes in response to diverse stimuli such as light, pH, temperature, solvation, metal ions, and mechanical stress.[97,98] Spiropyran undergoes a reversible photocleavage of the spiro C-O bond, which allows switching between a ring-closed, colorless, hydrophobic spiro form (SP) and a ring-open, colored, hydrophilic merocyanine form (MC). These compounds are converted to the colored MC form upon exposure to UV light, but revert to the colorless spirocyclic form upon irradiation with visible light.[99,100] However, to fully exploit this unique behavior, spiropyran must be covalently immobilized to a suitable scaffold without restricting its structural transformation. Tethering spiropyran to a support prevents leaching, improves processability, solubility, biocompatibility, minimizes photodegradation, control the structural change, and improves fluorescence.[97] Hence, developing scaffolds that spiropyran can attach onto has research value.

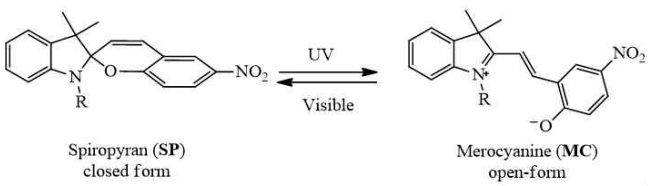

Figure 17. The reversible spiropyran-merocyanine isomerization in response to light irradiation.

Recently, the incorporation of interpenetrating polymer network (IPN) structures into pre-formed hydrogels has generated a lot of interest because of their potential application in the development of protein-resistant substrates, drug delivery systems, and biomedical

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devices.[101,102] An IPN consists of two or more interlaced polymers that are not covalently bonded to each other. Therefore, by controlling the polymer composition and crosslinking method, the properties of the IPN structures can be tailored to obtain the desired features.[98,103] Especially, chitosans have been combined with specific stimuli-responsive polymers in IPN structures to make them enzyme-, pH-, and temperature sensitive.[104] The chitosan-based IPNs have been found to be suitable for drug-delivery systems and tissue-engineering hydrogels because of their excellent biocompatibility, biodegradability, and facile synthesis.[105,106]

In this study, multi-responsive hydrogels have been prepared using chitosan-based IPN structures. The pre-formed poly(hydroxyethyl methacrylate) (pHEMA) hydrogels were modified with chitosan-IPN structures which were newly synthesized using methacrylamide chitosan polymers. The subsequent functionalization with SP derivatives produced photochromic hydrogels and their photophysical properties such as color, absorption, and fluorescence were investigated upon exposure to specific illuminating sources and acid/base gases. Attaching spiropyran on the surface of the IPN hydrogel ensures maximum exposure to the stimuli and provides enough space for the reversible spiropyran-merocyanine molecular switch.

2. Experimental

2-Hydroxyethyl methacrylate (HEMA) was purchased from Junsei (Japan). Ethylene glycol dimethacrylate (EGDMA), Azobisisobutyronitrile (AIBN), and ammonium persulfate (APS) were acquired from Sigma-Aldrich. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloric acid (EDC.HCl) and sodium metabisulfite (SMBS) were sourced from Daejung. N-hydroxysuccinimide (NHS) and methacrylic anhydride were purchased from Alfa Aesar. Chitosan (600-800 kDa) was procured from Acros Organics (Geel, Belgium). Concentrated hydrochloric acid, ammonia solution (7 N in methanol), dimethylformamide (DMF), tetrahydrofuran (THF), chloroform, and other reagents were of analytical grade and used without further purification. Methacrylamide chitosan and carboxylic acid containing SP (SP-COOH) were synthesized according to previously reported procedures.[90,107] The absorbance and fluorescence of the hydrogels was determined by using the Shimadzu UV-1650 PC (Japan) spectrophotometer and the Hitachi F-7000 fluorescence spectrophotometer, respectively. The hydrogels were illuminated with UV-light at 365 nm (UV-Model LV, France; 8 mW cm-2) or white LED light (SM 400-1,Korea;48W).

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2.1 Synthesis of SP-functionalized IPN Hydrogel

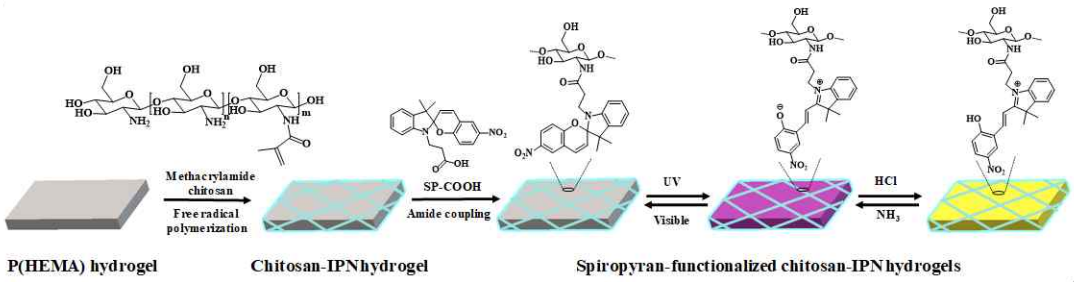

Figure 18. Preparation of SP-functionalized chitosan-IPN hydrogels and the structural conversion of SP with light and gases.

EGDMA (0.04 g) and a small amount of azobisisobutyronitrile were dissolved in HEMA (9.92 g). The solution was sonicated for 10 min and transferred to a square mold made of two glass plates covered internally with a polypropylene sheet. The molds were exposed to UV light for 30 min and heated at 80 ° C for at least 4 h. Subsequently, the samples were removed from the molds and soaked in distilled water for 2 days to remove any unreacted reagents. After drying overnight at 40 ° C, they were immersed in a solution of methacrylamide chitosan in distilled water (2 w/v %) for 24 h. The samples were then washed with distilled water and immersed in 10 mL of a solution of APS (5 mg) and SMBS (5 mg) for 24 h. The hydrogels were washed with distilled water and immersed in THF with EDC, NHS, and SP-COOH for 24 h. Finally, they were washed with ethanol and distilled water for 2 days to remove any unreacted chemicals prior to characterization and to allow swelling, which facilitated cutting.[108] The resulting hydrogels were cut into 1×1 cm samples.

2.2 Isomerization of spiropyran in response to illumination and acid/base gas treatment

The hydrogels were illuminated either with white LED light for a minute or UV-light at 365 nm for three minutes to evaluate their photochromic ability. The portable light source was held 5 cm above the hydrogels. To observe their response to acid and base treatment, the purple hydrogels were placed in such a way that they completely covered the opening of the vials containing 4 mL of conc. hydrochloric acid (3 min) and 7 M ammonia in methanol solution (5 min), respectively.

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3. Results and discussion

The multi-responsive hydrogels were prepared according to the synthetic scheme shown in Figure 18. In the first step, p(HEMA)-based hydrogels were prepared via radical polymerization using HEMA monomer with EGDMA as a cross-linker. In the second step, the resulting p(HEMA)-based hydrogels were interpenetrated with the methacrylamide chitosan polymer, and subsequent radical polymerization between chitosan backbones resulted in the chitosan-IPN structures consisting of cross-linked chitosan and p(HEMA) networks. Finally, the chitosan-IPN structures were covalently modified with SP molecules to form the multi-stimuli responsive hydrogels. Since natural chitosan is usually soluble in acidic conditions, it must be chemically modified to increase its solubility in neutral aqueous media or common organic solvents to be employed for the construction of chitosan-IPN structures.[106] Herein, we introduced methacrylate functionalities onto the chitosan polymers, giving rise to a methacrylamide chitosan.[107] The degree of methacryl modification was calculated to be 67.48%, based on the integrated area of the H2-H6 peaks observed in the range of 2.8-4.0 ppm and the methylene peaks at 5.2 and 5.6 ppm. Notably, methacrylamide chitosan contains considerable amounts of amine groups within its backbones. The p(HEMA) hydrogel modified with chitosan-IPN structures (**C-IPN-H**) was functionalized with SP-COOH via amide coupling between the primary amines in the chitosan backbones and the carboxylic acid groups of SP-COOH, resulting in the desired SP-functionalized hydrogel (**SP-IPN-H**).

To quantify the amount of SP molecules on the resultant hydrogels, UV/Vis absorption measurements were carried out using a standard calibration curve based on free SP-COOH in solution in a concentration range of 0.1 to 0.4 mM. Using Beer's Law and performing linear regression at 336 nm, the quantity of SP molecules incorporated on the hydrogel surface was estimated to be 0.140 ± 0.0196 μmol/cm^2. UV/Vis absorption and emission spectra were measured to investigate the photochromic properties in response to exposure to UV/Vis irradiation. The SP-modified hydrogel showed a strong absorption band in the range of 200 to 380 nm before exposure to UV radiation, while no absorbance was detected above 450 nm. This was attributed to the ring-closed SP form, which was predominant in the hydrogels. Upon UV-irradiation at 365 nm for 3 min, the SP-modified hydrogels changed from colorless to purple. Meanwhile, a new absorption band centered at 550 nm emerged because of the formation of the ring-open MC isomer with larger π-electron delocalization in the molecule (Fig. 19 b).[97] This was accompanied by a strong red fluorescence emission at ~630 nm at 347 nm excitation, resulting from the structural-conversion to the MC state (Fig. 19 c). These observations were in quite good accordance with those reported in the literature, wherein the water contact angles of photochromic hydrogel

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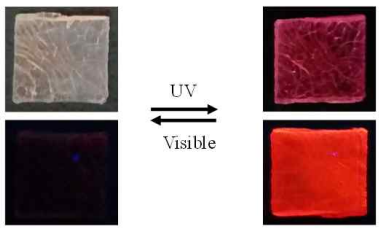
surfaces with MC state isomers decreased relative to those of SP isomers, showing that the surface wettability of the photochromic hydrogel is controllable by light.[109]



Picture 3. Visual changes to the sample upon irradiation of 365 nm UV light for 3 min.

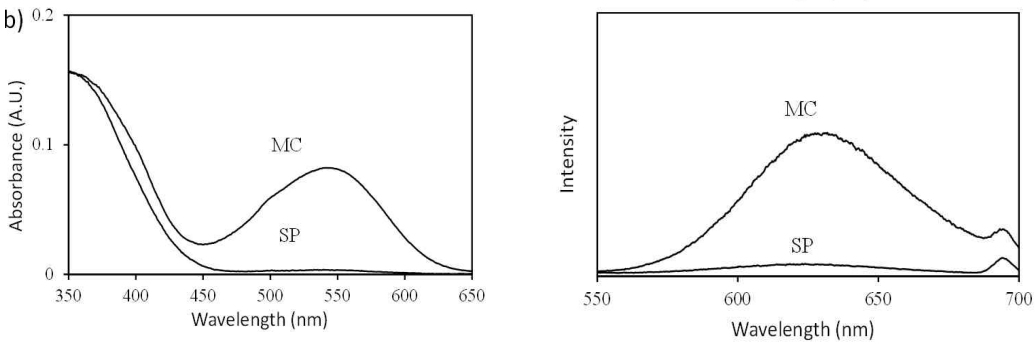


Figure 19. Absorbance spectra of **SP-IPN-H** before and after UV irradiation (left). Fluorescence spectra illustrating the isomerization of SP and MC (right) (λ_{ex} = 347nm).

The reverse photo-responsive isomerization of SP molecules in the hydrogels was investigated under white LED irradiation. After exposure to LED light, the purple hydrogels turned colorless and the distinct absorbance peak at 550 nm disappeared, suggesting that the ring-closed SP form was predominant, thus demonstrating their photochromic reversibility. The resultant colorless hydrogel also showed a decreased emission peak at 630 nm when excited at 347 nm, indicative of the formation of the SP form (Fig. 21c).

Figure 21b shows the UV/Vis spectra of the hydrogels acidified with HCl vapor for 3 min and stored in the dark. The purple hydrogel gradually

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faded and turned dime-yellow in color corresponding to the strong absorbance band with peaks at ~400-470 nm. This decolorization was attributed to the protonated form of MC, MCH, which is a stable ring-open isomer formed under acidic conditions.[94] The hydrogel with the MCH form was non-fluorescent under UV light because the protonation of the phenolate ion in the MC form significantly decreased the efficiency of fluorescence emission.

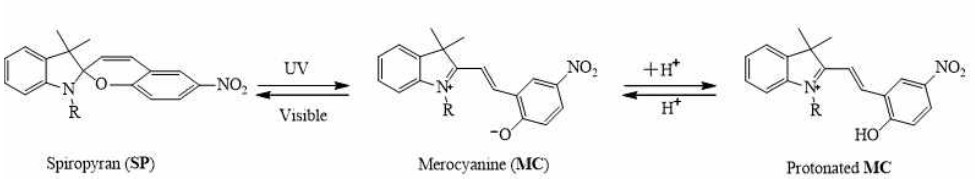
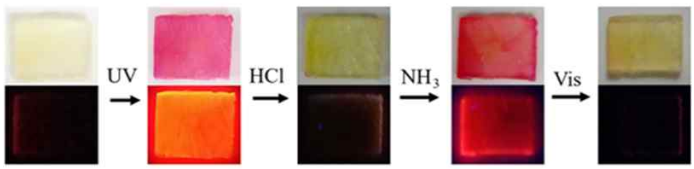


Figure 20. The changes in the spiropyran structure with light irradiation and exposure to acid/base gases.



Picture 4. Visual changes in **SP-IPN-H** as it responded to light and HCl/NH_3 gases.

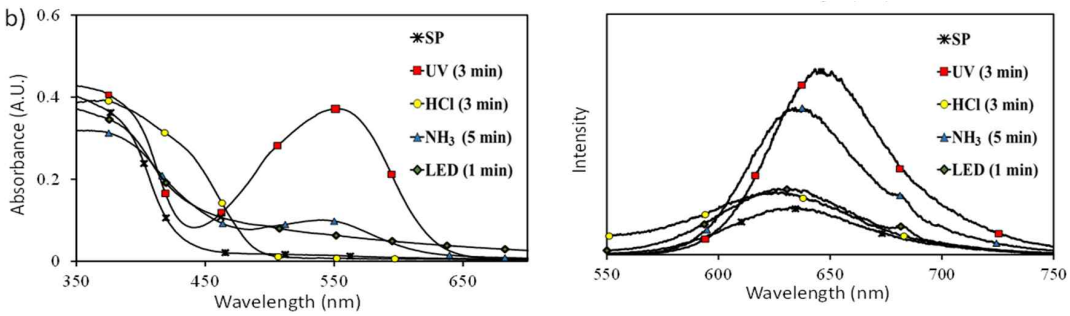


Figure 21. Corresponding absorbance spectra of **SP-IPN-H** (left). Fluorescence spectra of **SP-IPN-H** (right) (λ_{ex} = 347nm).

By neutralizing the acidic hydrogels with NH_3 gas,the hydrogel, which had been yellowish under acidic conditions, turned purple within 5 min,

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and regained its former color within a moment when acidified by treatment with HCl gas. Usually, photochromic materials that isomerize in response to stimuli, including SP derivatives, need enough space to change their structures.[110] Herein, chitosan-IPN structures on p(HEMA) hydrogels proved to be an excellent matrix for the spatial structural changes of the SP molecules. The surface-immobilized SP molecules were able to interchange between their isomers, producing photochromic and fluorescent hydrogels. Furthermore, the protonation and deprotonation processes of the hydrogel that resulted in the color changes could be performed at least five times.

4. Conclusions

In summary, we developed a novel method leading to the formation of multi-responsive hydrogels that changed their color and fluorescence upon UV illumination or acid-base treatment. The p(HEMA)-based hydrogels were synthesized first and then interpenetrated with methacrylamide chitosan polymers. The cross-linking reactions between the chitosan backbones yielded the chitosan-IPN structures consisting of chitosan networks and the p(HEMA) matrix. Further SP-modification resulted in the multi-responsive photochromic hydrogels. With UV irradiation, the colorless SP on the hydrogel isomerized to its purple fluorescent MC form. In addition, the MC form could be protonated by treatment with HCl vapor, resulting in a yellow hydrogel. This acid-derived process, in turn, could be reversed by NH_3 treatment, giving rise to a purple hydrogel. The results described herein provide a useful strategy to develop photochromic optical devices and chemical sensors.

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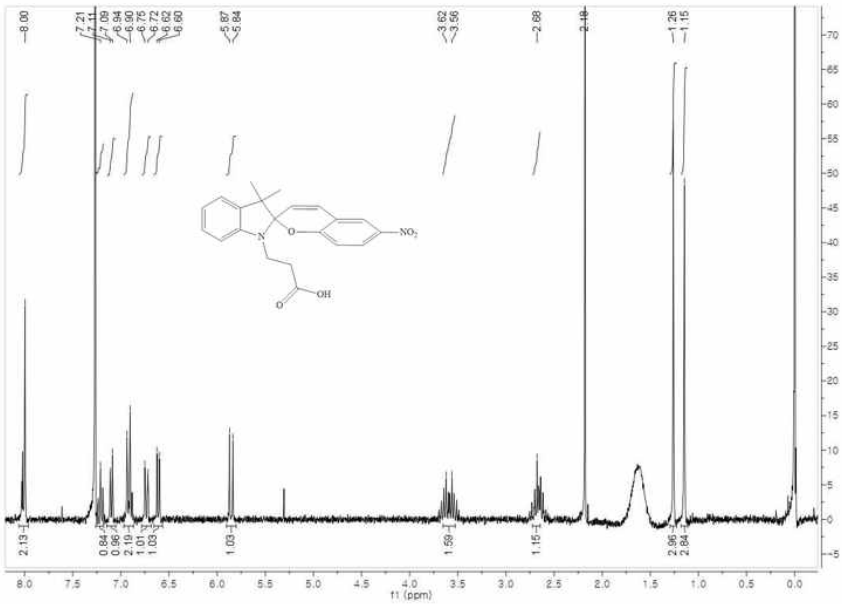
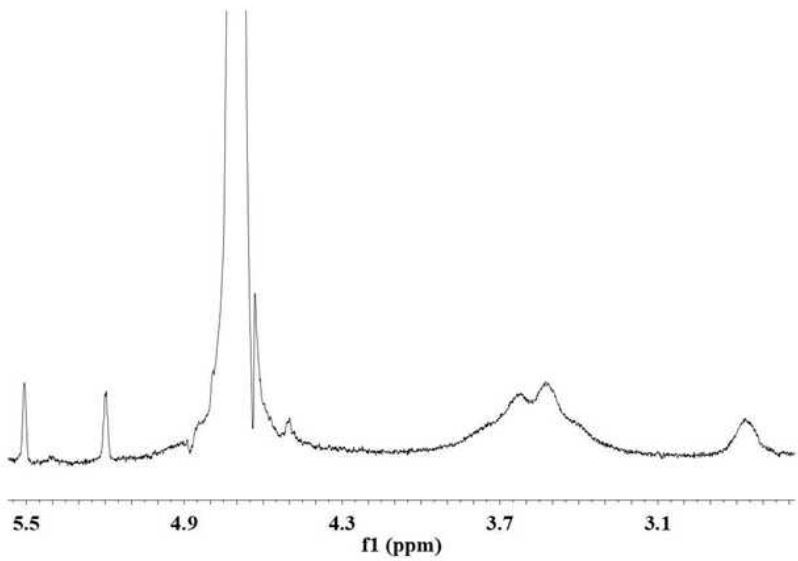


^{1}H NMR of Spiropyran-propionic acid



^{1}H NMR of methacrylamide chitosan

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