

**SYNTHESIS AND CHARACTERIZATION OF NEAR-
INFRARED (NIR) EMISSIVE CONJUGATED POLYMER
DOTS FOR TUMOROID IMAGING**

**A Thesis Submitted to
the Graduate School of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
DOCTOR OF PHILOSOPHY
in Chemistry**

**by
Soner KARABACAK**

**July 2024
İZMİR**

We approve the thesis of **Soner KARABACAK**

Examining committee members:

Assoc. Prof. Dr. Ümit Hakan YILDIZ

Department of Chemistry, İzmir Institute of Technology

Prof. Dr. Talat Yalçın

Department of Chemistry, İzmir Institute of Technology

Assoc. Prof. Dr. Ümit Tayfun

Department of Basic Sciences, Bartın University

Assist. Prof. Dr. Onur BÜYÜKÇAKIR

Department of Chemistry, İzmir Institute of Technology

Prof. Dr. Armağan KINAL

Department of Chemistry, Ege University

05 July 2024

Assoc. Prof. Dr. Ümit Hakan YILDIZ

Supervisor, Department of Chemistry,
İzmir Institute of Technology

Prof. Dr. Gülşah ŞANLI MOHAMED

Head of the Department of Chemistry

Prof. Dr. Mehtap EANES

Dean of the Graduate School

ACKNOWLEDGEMENTS

Firstly, I would like to express my deep gratitude to my advisor, Assoc. Prof. Dr. Ümit Hakan YILDIZ, for his expert guidance, valuable recommendations, motivation, friendliness, and patience. His support has been invaluable not just during this journey but also in every aspect of my life. Additionally, I would like to express my gratitude to Assoc. Prof. Dr. Ahu ARSLAN YILDIZ for her invaluable assistance concerning my thesis. I also thank my thesis committee members Prof. Dr. Talat YALÇIN, and Assoc. Prof. Ümit TAYFUN for their participation and valuable advice.

I would like to express my gratitude to my lovely wife Bahar DEMİRTAŞ KARABACAK for her unwavering support, intellectual contributions, and encouragement throughout my doctoral studies and in my personal life. I would like to extend my appreciation to my mother, Ayşe KARABACAK, as well as my siblings and other cherished relatives, for their unwavering support and affection during every minute of my life.

I would like to thank Başak ÇOBAN and Tolunay KURT for their assistance with this thesis. I am also grateful to Prof. Dr. Mustafa EMRULLAHOĞLU for enabling us to benefit from the laboratory facilities and Ahmet EREN's assistance. I would like to express my gratitude to the members of the Biomacros Research Group for their assistance and friendship. Additionally, I would like to express my gratitude to all alumni for their assistance and encouragement.

This study was funded by the Scientific and Technological Research Council of Turkey (TUBİTAK) under project 120Z588. In addition, I would like to express my gratitude for the scholarship provided by TUBİTAK Directorate of Science Fellowships and Grant Programmes (BİDEB) 2211-A program for Ph.D. and the Council of Higher Education (CoHE) in Optic and Photonics Technologies as part of the 100/2000 Program by CoHE.

ABSTRACT

SYNTHESIS AND CHARACTERIZATION OF NEAR-INFRARED (NIR) EMISSIVE CONJUGATED POLYMER DOTS FOR TUMOROID IMAGING

This thesis describes the synthesis and characterization of near-infrared (NIR) emissive conjugated polymers and their polymer dots (Pdots). The Pdots were exploited to image the tumor cells and tumor spheroids. The penetration behavior of NIR emissive Pdots was characterized in five different tumor spheroid models. Three different polymerization techniques were tried to synthesize the NIR emissive polymers, namely oxidative, direct arylation, and Stille polymerization. The obtained NIR emissive polymers underwent structural and optical characterization. P1 was chosen as a model polymer to obtain Pdots from NIR emissive polymers for imaging tumoroids. Pdot preparation includes using ultrasonic emulsification to modify nonionic D-A-D type alkoxy thiophene-benzobisthiadiazole-based conjugated polymers (P1) with amphiphilic cetyltrimethylammonium bromide (CTAB). The technique yields Pdots with a significant positive surface charge of $+56.5 \text{ mV} \pm 9.5$ and an average hydrodynamic radius of 12 nm. Optical characterization reveals that these Pdots were found as emissive in the NIR region, with a maximum wavelength of 860 nm. These Pdots possess colloidal and optical properties that make them appropriate for use as fluorescence emissive probes in bioimaging applications. The advantageous use of positively charged Pdots has been proven in diffusion-limited settings such as tissues, specifically in certain tumor spheroid models produced from the tumoroid cell lines. After the fluorescence imaging analysis, the Pdots' emission intensity profile indicates that they have high penetration capability into the tumoroid models' center parts. The results show that Pdots with a single-chain donor-acceptor polymer structure that has been cationized with CTAB can penetrate through dense materials over about 1 μm . This provides valuable insights into the progression of targeted theranostic strategies in cancer therapy.

ÖZET

TÜMÖROİD GÖRÜNTÜLEME İÇİN YAKIN KIZILÖTESİ (NIR) IŞIYAN KONJUGE POLİMER NOKTALARININ SENTEZİ VE KARAKTERİZASYONU

Bu tez, yakın kızılötesi (NIR) ışıyan konjuge polimerlerin ve bunların polimer noktalarının (Pdot'lar) sentezini ve karakterizasyonunu açıklamaktadır. Pdot'lar, tümör hücrelerini ve tümör sferoidlerini görüntülemek için kullanıldı. NIR ışıyan Pdot'ların penetrasyon davranışı farklı tümör sferoid modellerinde karakterize edildi. NIR ışıyan polimerleri sentezlemek için oksidatif, direkt arilasyon ve Stille polimerizasyonu olmak üzere üç farklı polimerizasyon tekniği denendi. Elde edilen NIR ışıyan polimerlere yapısal ve optik karakterizasyon uygulandı. P1, tümörlerin görüntülenmesi için NIR ışıyan polimerlerden Pdot'lar elde etmek amacıyla model polimer olarak seçildi. Pdot hazırlığı, iyonik olmayan D-A-D tipi alkoksi tiyofen-benzobistiyadiazol bazlı konjuge polimerleri (P1) amfifilik setiltrimetilamonyum bromür (CTAB) ile modifiye etmek için ultrasonik emülsifikasyonun kullanılmasını içerir. Teknik, $+56,5 \text{ mV} \pm 9,5$ önemli pozitif yüzey yüküne ve 12 nm ortalama hidrodinamik yarıçapa sahip Pdot'lar sağlar. Optik karakterizasyon, bu Pdot'ların NIR bölgesinde maksimum dalga boyu 860 nm olan ışıyan olarak bulunduğunu ortaya koymaktadır. Bu Pdot'lar, onları biyogörüntüleme uygulamalarında floresans ışıyan proplar olarak kullanıma uygun kılan kolloidal ve optik özelliklere sahiptir. Pozitif yüklü Pdot'ların avantajlı kullanımı, dokular gibi difüzyonun sınırlı olduğu ortamlarda, özellikle tümöroid hücre hatlarından üretilen belirli tümör sferoid modellerinde kanıtlanmıştır. Floresan görüntüleme analizinden sonra, Pdot'ların emisyon yoğunluğu profili, tümöroid modellerinin merkez kısımlarına yüksek nüfuz etme kapasitesine sahip olduklarını göstermektedir. Sonuçlar, CTAB ile katyonize edilmiş tek zincirli donör-alıcı polimer yapısına sahip Pdot'ların, yaklaşık 1 μm 'nin üzerinde yoğun malzemelere nüfuz edebildiğini göstermektedir. Bu, kanser tedavisinde hedefe yönelik teranostik stratejilerin ilerlemesine ilişkin değerli bilgiler sağlar.



Dedicated to my mother...

TABLE OF CONTENTS

LIST OF FIGURES	ix
LIST OF TABLES	xv
CHAPTER 1 INTRODUCTION	1
1.1. Motivation and scope of the thesis.....	1
1.2. NIR emissive conjugated polymers	4
1.3. Polymer dots	9
1.4. Literature Works for NIR emissive Pdots.....	13
CHAPTER 2 MATERIALS AND METHODS	17
2.1. Materials	17
2.2. Synthesis of donor-acceptor-donor based monomers	18
2.2.1. Synthesis of Monomer 1	18
2.2.2. Synthesis of Monomer 2	21
2.2.3. Synthesis of Monomer 3	23
2.3. Synthesis of NIR Emissive Conjugated Polymers.....	24
2.3.1. Oxidative Polymerization	24
2.3.2. Direct arylation polymerization	26
2.3.3. Stille Polymerization.....	29
2.4. Preparation of the Pdots	32
2.5. Characterization Methods	33
2.6. Fluorescence imaging application with Pdots in 2D and 3D cell culture models.....	36
2.7. Karhunen-Loève Transform (KLT) Analysis	40
CHAPTER 3 RESULTS AND DISCUSSIONS	41
3.1. Characterization of donor-acceptor-donor based monomers.....	41
3.1.1. Characterization of M1	42
3.1.2. Characterization of M2	46
3.1.3. Characterization of M3	50

3.2. Characterization of NIR emissive conjugated polymers	53
3.2.1. Oxidative Polymerization Products	55
3.2.2. Direct Arylation Polymerization Products.....	59
3.2.3. Stille Polymerization Products.....	63
3.3. Characterization of Pdots	73
3.4. Optimization and Characterization of imaging parameters with Pdots in standard 2D cell culture models	82
3.5. Optimization and Characterization of imaging parameters with Pdots in standard 3D cell culture models	87
CHAPTER 4 CONCLUSION	108
REFERENCES	111

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
Figure 1.1. Timeline for this thesis.	3
Figure 1.2. Representation of the analyzing Pdots's penetration ability into the tumoroid spheroids with a comparement with DAPI.	4
Figure 1.3. Chemical structures of D–A–D based conjugated polymers for Pdots.	6
Figure 1.4. Mechanism of the typical oxidative polymerization.	7
Figure 1.5. Mechanism of typical DArP reactions.	8
Figure 1.6. Mechanism of typical Stille polymerization.	9
Figure 1.7. Nanoprecipitation method for preparation of Pdot.	11
Figure 1.8. Miniemulsion method for preparation of Pdot.	11
Figure 1.9. Preparation of polymer dots using solid support.	12
Figure 1.10. Schematic illustration of the nanophase separation method for preparation of Pdot.	13
Figure 2.1. Photograph of the a) dry ice machine, and b) reaction medium that is controlled by the thermometer.	20
Figure 2.2. Synthesis protocol for M1.	21
Figure 2.3. Synthesis protocol for M2.	22
Figure 2.4. Synthesis protocol for M3.	24
Figure 2.5. Oxidative polymerization protocol for P1.	25
Figure 2.6. Oxidative polymerization protocol for P2.	25
Figure 2.7. Oxidative polymerization protocol for P3.	26
Figure 2.8. DArP protocols for P BuMT.	28
Figure 2.9. DArP protocols for P4.	28
Figure 2.10. DArP protocol for P5.	29
Figure 2.11. Synthesis protocol for two-sided stannylated DiBuT.	30
Figure 2.12. Synthesis protocol for two-sided stannylated EDOT.	31
Figure 2.13. Stille polymerization protocols for P6-10 (P7-8-9 NIR emissive polymer structure were given with content ratios of the donor compounds).	32
Figure 2.14. Photograph of the ultrasonicator.	33
Figure 2.15. Schematic illustration for the preparation of NIR emissive Pdots.	33

<u>Figure</u>	<u>Page</u>
Figure 2.16. Varian NMR instrument (400 MHz).	34
Figure 2.17. Horiba Duetta absorbance and emission spectrometer.....	34
Figure 2.18. Zetasizer Nano ZS, Malvern Instruments.....	35
Figure 2.19. Nanosurf-Core AFM instrument.	35
Figure 2.20. Fluorescence microscopy instrument.	36
Figure 2.21. Screen shot of the FIJI Color Inspector 3D (v2.5) and KLT analysis cell spheroids.....	40
Figure 3.1. ^1H NMR spectrum of BuMT.	42
Figure 3.2. ^{13}C NMR spectrum of BuMT.	43
Figure 3.3. ^1H NMR spectrum of M1.	44
Figure 3.4. ^{13}C NMR spectrum of M1.	44
Figure 3.5. MS spectrum of M1.....	45
Figure 3.6. Absorption and emission spectra of D-A-D M1 in chloroform.	46
Figure 3.7. ^1H NMR spectrum of DiBuT.....	47
Figure 3.8. ^{13}C NMR spectrum of DiBuT.	47
Figure 3.9. ^1H NMR spectrum of one-sided stannylated DiBuT.....	48
Figure 3.10. ^1H NMR spectrum of Monomer 2.....	49
Figure 3.11. ^{13}C NMR spectrum of the M2.	49
Figure 3.12. Absorption and Emission spectra of M2 in chloroform.	50
Figure 3.13. ^1H NMR spectrum of one-sided stannylated EDOT.	51
Figure 3.14. ^1H NMR spectrum of Monomer 3.....	52
Figure 3.15. Absorption and emission spectra of M3 in chloroform.....	52
Figure 3.16. Stack ^1H NMR spectrum of NIR emissive P1 with M1.	55
Figure 3.17. Absorption and emission spectra of NIR emissive P1 in chloroform.	56
Figure 3.18. Absorption and emission spectrum of P2 in chloroform.....	57
Figure 3.19. Absorption and emission spectrum of P3 in chloroform (excitation wavelength: 900 nm).	58
Figure 3.20. Comparison of absorption spectra between oxidative polymerization products (P1, P2, and P3).	59
Figure 3.21. ^1H NMR spectrum of the Br-BUMT.....	60
Figure 3.22. Stacked ^1H NMR spectra for PBUtMT via three different DArP protocols.	61

<u>Figure</u>	<u>Page</u>
Figure 3.23. Absorption spectrum of PBUtMT via three different DArP protocols.	62
Figure 3.24. Absorption spectra for the P4 via three different DArP protocols.	63
Figure 3.25. ¹ H NMR spectrum of the two-sided stannylated EDOT.	64
Figure 3.26. ¹ H NMR spectrum of the two-sided stannylated DiBuT.	65
Figure 3.27. Absorption and emission spectrum of P6 in chloroform (excitation wavelength: 700 nm).	66
Figure 3.28. Absorption and emission spectrum of P7 in chloroform (excitation wavelength: 700 nm).	67
Figure 3.29. Absorption and emission spectrum of P8 in chloroform (excitation wavelength: 700 nm).	68
Figure 3.30. Absorption and emission spectrum of P9 in chloroform (excitation wavelength: 650 nm).	69
Figure 3.31. Absorption and emission spectrum of P10 in chloroform (excitation wavelength: 630 nm).	70
Figure 3.32. Absorbance spectra for all Stille polymerization products in chloroform.	71
Figure 3.33. Emission spectra for all Stille polymerization products in chloroform (excitation wavelength: 650 nm).	72
Figure 3.34. Photograph of the Stille polymerization products (P6–10) in chloroform under long wave UV light.	73
Figure 3.35. (A) Synthesis protocol of NIR emissive P1, (B) schematic representation of the preparation of Pdots.	74
Figure 3.36. Absorption and emission spectra of NIR emissive Pdot in water.	75
Figure 3.37. The graph of $\ln(A/A_0)$ versus time for the NIR emissive Pdot.	76
Figure 3.38. Analysis of Pdots containing NIR emissive polymers in solution with size measurements for pure CTAB micelles and NIR emissive Pdot.	76
Figure 3.39. Analysis of pure CTAB nanoparticles (NPs) and the Pdots containing NIR emissive polymers in solution with the number-averaged size measurements.	77
Figure 3.40. (A) Size values at different time points and pH range (5.5–7.5) graph for the NIR emissive Pdot; (B) Zeta potential values at different time points and pH range (5.5–7.5) graph for the NIR emissive Pdot.	78
Figure 3.41. SEM image of NIR emissive Pdot.	79

<u>Figure</u>	<u>Page</u>
Figure 3.42. A) Topography images and cross-section analysis of the Pdots (Pdot1-6 bottom of this part) by AFM (scale bars for AFM images: 2µm, 200nm, and 50nm, respectively).....	80
Figure 3.43. Size analysis, and auto-correlation graphs of NIR emissive Pdot by DLS with a photograph of the Pdot solution in water.....	80
Figure 3.44. EFM topography images of NIR emissive Pdot at different voltage states and phase degree graphs respectively.....	81
Figure 3.45. Fluorescence microscopy images of MCF-7, SH-SY5Y, PC-12, HeLa, and MDA-MB-23 cells, cultured with Pdot and labeled with DAPI and Calcein Green (blue: DAPI, red: Pdot, green: Calcein, scale bar: 20 µm).	83
Figure 3.46. Fluorescence microscopy images of MCF-7 cells cultured with Pdot for 3, 5, and 7 days labeled with DAPI and Calcein Green (blue: DAPI, red: Pdot, green: Calcein, scale bar: 20 µm).	84
Figure 3.47. Fluorescence microscopy images of SH-SY5Y cells cultured with Pdot for 3, 5, and 7 days labeled with DAPI and Calcein Green (blue: DAPI, red: Pdot, green: Calcein, scale bar: 20 µm).	84
Figure 3.48. Fluorescence microscopy images of PC-12 cells cultured with Pdot for 3, 5, and 7 days labeled with DAPI and Calcein Green (blue: DAPI, red: Pdot, green: Calcein, scale bar: 20 µm).	85
Figure 3.49. Fluorescence microscopy images of MCF-7, SH-SY5Y, PC-12, HeLa, and MDA-MB-23 cells, cultured with Pdot and labeled with DAPI and MitoView (blue: DAPI, red: Pdot, green: MitoView, scale bar: 20 µm)..	86
Figure 3.50. Relative Cell viability results obtained from Alamar blue analysis of Pdot cytotoxicity on MCF-7, SH-SY5Y, PC-12, HeLa, and MDA-MB-231 cells.....	87
Figure 3.51. Light microscope analysis images of MCF-7 spheroids using 25×10^3 and 50×10^3 cell numbers produced by the hanging drop method. (Scale bar: 200 µm).	88
Figure 3.52. Light microscope analysis images of SH-SY5Y spheroids using 25×10^3 and 50×10^3 cell numbers produced by the hanging drop method. (Scale bar: 200 µm).	89

<u>Figure</u>	<u>Page</u>
Figure 3.53. Light microscope analysis images of PC-12 spheroids using 25×10^3 and 50×10^3 cell numbers produced by the hanging drop method. (Scale bar: 200 μm).	89
Figure 3.54. Light microscopy analysis images of HeLa spheroids using a cell count of 25×10^3 and 50×10^3 produced by the hanging drop method. (Scale bar: 200 μm).	90
Figure 3.55. Light microscopy analysis images of HeLa spheroids using a cell count of 10×10^3 produced by the hanging drop method. (Scale bar: 200 μm). ..	90
Figure 3.56. Light microscope analysis images of MDA-MB-231 spheroids using 25×10^3 and 50×10^3 cell numbers produced by the hanging drop method. (Scale bar: 200 μm).	91
Figure 3.57. Fluorescence microscope image of Pdot-treated MCF-7, SH-SY5Y, HeLa and MDA-MB-231 spheroid in 96 microplates (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 100 μm).	92
Figure 3.58. Roundness analysis of HeLa, MDA-MB-231, MCF-7 and SH-SY5Y spheroids performed by fluorescence microscopy analysis in 96 microplates.....	94
Figure 3.59. Roundness analysis of HeLa, MDA-MB-231, MCF-7, SH-SY5Y and PC-12 spheroids performed by fluorescence microscopy analysis on a glass surface.....	95
Figure 3.60. A) Fluorescence microscope image of 3D MCF-7 spheroid with Pdot applied on glass surface (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 200 μm). B) Pdot and DAPI penetration behavior along the diameter of the Pdot-treated MCF-7 spheroid.....	96
Figure 3.61. A) Fluorescence microscope image of 3D SHSY-5Y spheroid with Pdot applied on glass surface (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 200 μm). B) Pdot and DAPI penetration behavior along the diameter of the Pdot-treated SHSY-5Y spheroid.	97
Figure 3.62. A) Fluorescence microscope image of 3D PC-12 spheroid with Pdot applied on glass surface (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 200 μm). B) Pdot and DAPI penetration behavior along the diameter of the Pdot-treated PC-12 spheroid.	98

<u>Figure</u>	<u>Page</u>
Figure 3.63. A) Fluorescence microscope image of 3D MDA-MB-231 spheroid with Pdot applied on glass surface (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 200 μ m). B) Pdot and DAPI penetration behavior along the diameter of the Pdot-treated MDA-MB-231 spheroid.....	100
Figure 3.64. A) Fluorescence microscope image of 3D HeLa spheroid with Pdot applied on glass surface (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 200 μ m). B) Pdot and DAPI penetration behavior along the diameter of the Pdot-treated HeLa spheroid.....	102
Figure 3.65. Fluorescence microscopy images of 3D MCF-7, SH-SY5Y, and PC-12 cell spheroids cultured with Pdot and labeled with MitoView Green (red: Pdot, green: MitoView, scale bar: 200 μ m).	103
Figure 3.66. Fluorescence intensity profile of Pdot and MitoView for MCF-7 cells...	104
Figure 3.67. Fluorescence intensity profile of Pdot and MitoView for SH-SY5Y cells.....	104
Figure 3.68. Fluorescence intensity profile of Pdot and MitoView for PC-12 cells. ...	105
Figure 3.69. Pdot distribution and colocalization of Mitoview analysis with KLT/PCA for MCF-7, SH-SY5Y, and PC-12 cell spheroids.	106

LIST OF TABLES

<u>Table</u>	<u>Page</u>
Table 2.1. Synthesis information for the M1.	19
Table 2.2. Synthesis information for the M2.	22
Table 2.3. Synthesis information for the M3.	23
Table 2.4. Synthesis information for the oxidative polymerization products.	24
Table 2.5. Synthesis information for the DArP trials.	27
Table 2.6. Synthesis information for the Stille polymerization products.	30
Table 3.1. Synthesized Monomers.	41
Table 3.2. Synthesized NIR emissive conjugated polymers.	53
Table 3.3. Size, zeta potential (ZP), mobility (Mob), and conductivity (Cond) values for the NIR emissive Pdots at different pH values and time points (5.5– 7.5).	78
Table 3.4. Zeta potential measurement for NIR emissive Pdot.	81

CHAPTER 1

INTRODUCTION

1.1. Motivation and scope of the thesis

Alterations and distortions in tissues and their vicinities are quite significant and may indicate the onsets or progression of diseases.^{1,2} The present clinical setups that depend on microscopy-associated techniques produce compelling and comprehensive images, but they are limited by the resolution of the imaging methodology.^{3,4} The constraints in resolution, along with imaging aberrations, might potentially contribute to misleading results by introducing a higher degree of uncertainty that obscures micro-modifications in denser tissue. The main challenges in fluorescence microscopy imaging arise from the limited depth of penetration of electromagnetic radiation in the visible range (400–700 nm) and the presence of autofluorescence from fluorescent probes. Autofluorescence specifically results in emission occurring beyond the original position of probes, hence causing a mismatch with the desired region of interest. The requirement for optimization in fluorescence microscopy imaging modalities is obvious.^{5–7}

NIR fluorescent imaging has gained recent attention due to its ability to minimize interference absorption, reduce biological autofluorescence, and permit deep tissue penetration for biological applications compared to visible fluorescence imaging.^{8–11} So far, NIR emissive probes such as semiconducting polymers, rare earth-doped nanoparticles, quantum dots (CuInS/ZnS), single-walled carbon nanotubes, carbon dots, and metal nanoclusters have been documented.^{12–15} The findings provide evidence that NIR emissive probes have great potential in the fields of bioimaging, diagnostics, photodynamic treatment, and controlled drug delivery. This is mainly due to the extremely low intensity of autofluorescence.^{16–19} Within the range of molecular NIR probes, semiconducting polymers can undergo managed condensation/precipitation and produce nanoparticles known as "Pdots" that possess outstanding emissive and colloidal properties. Pdots, which are based on NIR emissive conjugated polymers, have

fluorescent characteristics that enable advanced bioimaging applications. These properties include high brightness, high extinction coefficients, and good photostability.^{20–24}

The size of Pdots is an important aspect that affects bioimaging techniques. For example, Pdots with a size of 50 nm tend to accumulate in the cytoplasm and lysosomes. On the other hand, Pdots with a size smaller than 20 nm can penetrate crowded cellular compartments such as mitochondria and microtubules near the nucleus.^{22,25} Therefore, these nanoscale Pdots offer enhanced tissue and subcellular penetration in biological investigations.²⁶ Nevertheless, according to recent statements, the process of creating Pdot particles with a diameter of approximately 10 nm is still difficult because of the instability of the colloidal solution as the size drops.²⁷ While traditional methods such as nanoprecipitation²⁸ and miniemulsion²¹ have been used to prepare NIR Pdots, extensive research on the relationship between particle size and penetration abilities is still lacking due to potential issues with colloidal instability.

Our research group has proposed a nanophase separation technique for preparing Pdots, which allows for the creation of extremely small-sized Pdots from positively charged semiconducting polymers.²⁹ Figure 1.1 depicts the utilization of conjugated polymer based Pdot, including their synthesis methods, optical properties, and application areas. The described nanophase separation approach yields monodisperse Pdots with a size of less than 10 nm. Özenler et al. demonstrated that cationic Pdots measuring 4 nm in size displayed enhanced nuclear penetration in cancer liver cells, enabling the differentiation between healthy and cancer cells. Yücel et al. then used cationic polythiophene Pdots to treat the breast cancer cell lines MCF-7 and MDA-MB-231, as well as their tumoroid models.³⁰ Fluorescence signals were obtained from the MDA-MB-231 tumoroid models to analyze the movement of Pdots in the triple-negative cancer cells. Nevertheless, nanophase separation is not appropriate for neutral (nonionic) semiconducting polymers^{31,32}, and the penetrating properties of Pdots have not been well investigated in dense mediums like tumoroids.^{33,34}

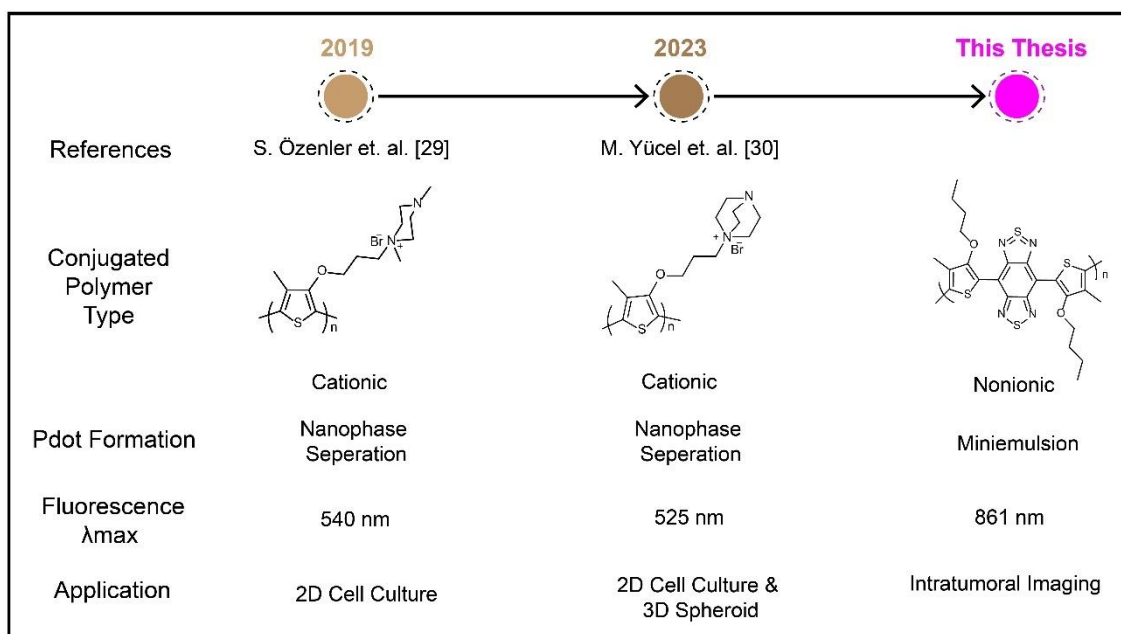


Figure 1.1. Timeline for this thesis.

This thesis focuses on synthesizing and characterizing conjugated polymers and associated Pdots that emit light in the NIR range. The objective is to examine the penetration capability of NIR emissive Pdots into both tumor cells and their spheroids. Three distinct polymerization procedures, i) oxidative, ii) direct arylation, and iii) Stille polymerization, were employed in attempts to produce NIR emissive polymers. The resulting NIR emissive polymers were subjected to structural and optical characterization. P1 was selected as a model polymer to generate Pdots from NIR emissive polymers to visualize tumoroids. A method was proposed for preparing and studying the penetration behavior of 12 nm cationic NIR Pdots. These Pdots are composed of a nonionic donor-acceptor-donor (D-A-D) type alkoxy thiophene and a benzobisthiadiazole-based conjugated polymer called poly(4,8-di-3-butoxy-4 methylthiophenebenzobisthiadiazole) (P1). The preparation involves emulsification assisted by sonication. The application of ultrasound in the presence of cationic surfactant, P1, produces energy to (mini)emulsify a low band gap D-A-D type water insoluble conjugated polymer, resulting in the production of positively charged Pdots. The fluorescence imaging technique was used to investigate the penetration behavior of the NIR emissive Pdots in all tumor models. The results demonstrated the efficient penetration of tumor structures by Pdots. Particularly, Pdots, known for their ability to

penetrate the inside of tumors, offer a crucial opportunity to visualize and inhibit tumor growth. Also, Pdots were used to analyze the penetration ability as compared to the commercial dye 4',6-diamidino-2-phenylindole (DAPI). The study was schematically drawn in Figure 1.2. This study shows the promise of utilizing these extremely small Pdots for imaging within tumors and promotes the need for additional research in the next years.

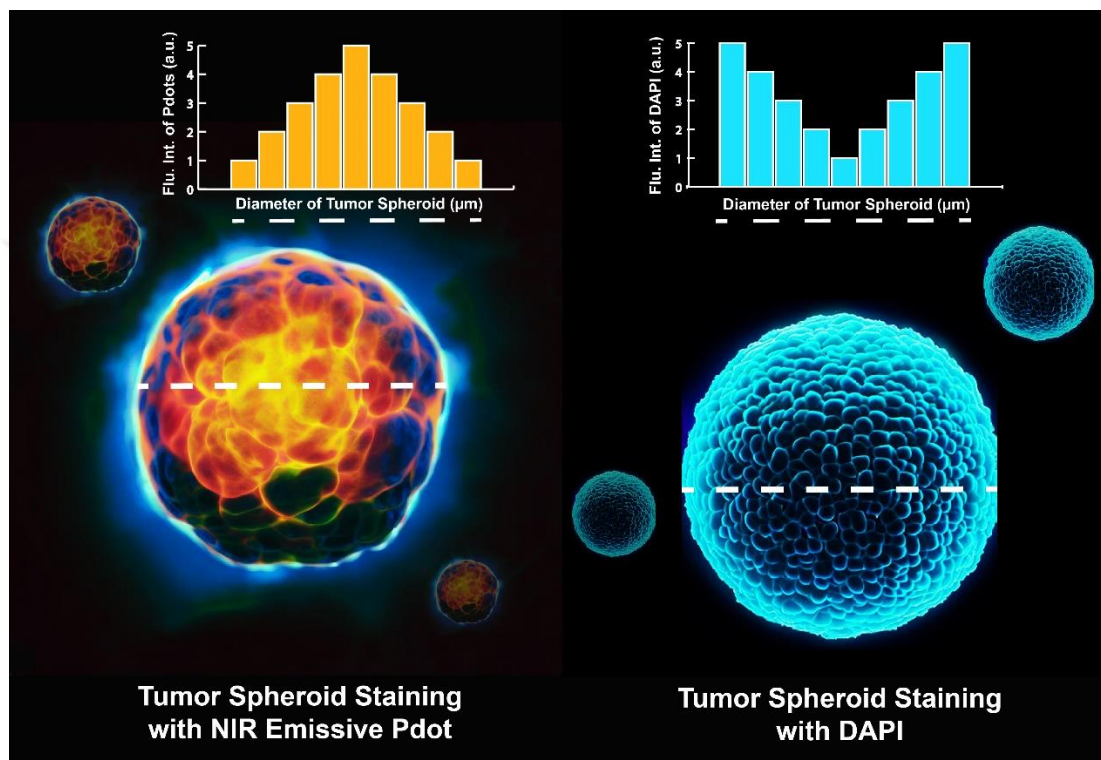


Figure 1.2. Representation of the analyzing Pdots's penetration ability into the tumoroid spheroids with a comparement with DAPI.

1.2. NIR emissive conjugated polymers

Advancements in polymer research have been remarkable in the last twenty years. Polymer-based products and novel technologies are becoming increasingly significant in various conventional areas, such as polymer solar cells (PSCs), organic field-effect transistors (OFETs), organic light-emitting diodes (OLEDs), and emerging areas in biomedicine. The enhanced ability of conjugated polymers to absorb and interact with biological systems makes them useful as flexible fluorescent molecules for

imaging biological processes. Modifying the D-A-D architecture of the molecules that provide energy allows for adjusting their ability to absorb and emit light.³⁵ This study provides a concise overview of the latest advancements in bioimaging employing conjugated polymers in the NIR windows. This text discusses the chemical synthesis, physiochemical properties, and bioimaging applications of fluorophores. It also proposes chemistry characteristics to create higher-quality fluorophores for enhanced NIR bioimaging.

Biological tissues frequently absorb and scatter visible light, greatly inhibiting their penetration.³⁶ Additionally, there is a significant presence of tissue autofluorescence in the visible spectrum, which poses a considerable obstacle to bioimaging. On the other hand, the NIR area significantly reduces the absorption and scattering of biological tissues, thereby increasing the opportunities for penetration and fluorescence.³⁷ In recent decades, there have been significant advancements and achievements in the field of synthesizing conjugated polymers for NIR imaging. These polymers offer distinct advantages over small fluorophore molecules, including a larger absorption coefficient and better exciton movement.^{38,39} Consequently, numerous polymer-based fluorophores have been produced and developed by various research groups globally to enable fluorescent bioimaging.^{40,41} Conjugated polymers are presently considered to be an excellent option for the next generation of fluorescent probes in bioimaging due to their improved photostability, resolution of images, and reduced cytotoxicity.⁴² Also, organic dyes⁴³, fluorescence proteins⁴⁴, and quantum dots⁴⁵ are often used in the visible and infrared regions via fluorescent properties in bioimaging applications. However, the probes have some limitations in their biological applications. For example, organic dyes and fluorescence proteins have small Stokes shifts since they can show self-absorption. Also, quantum dots have high toxicity in bioimaging studies. In recent years, conjugated polymers have become increasingly popular as an alternative to the other probes. Conjugated polymers have long π -conjugation on the backbone and modifiable functional groups to provide high quantum efficiency, as well as adjustable absorption and fluorescence emission properties.⁴⁶ In addition to the optical properties of conjugated polymers, they have unique properties such as good colloidal stability, high photostability, low toxicities, and especially the solubility of conjugated polymers in water, which is so useful for bioimaging⁴⁷, carrier

systems, and biosensors.⁴⁸ Figure 1.3 displays the chemical structures of the newly synthesized conjugated polymers based on D-A-D.

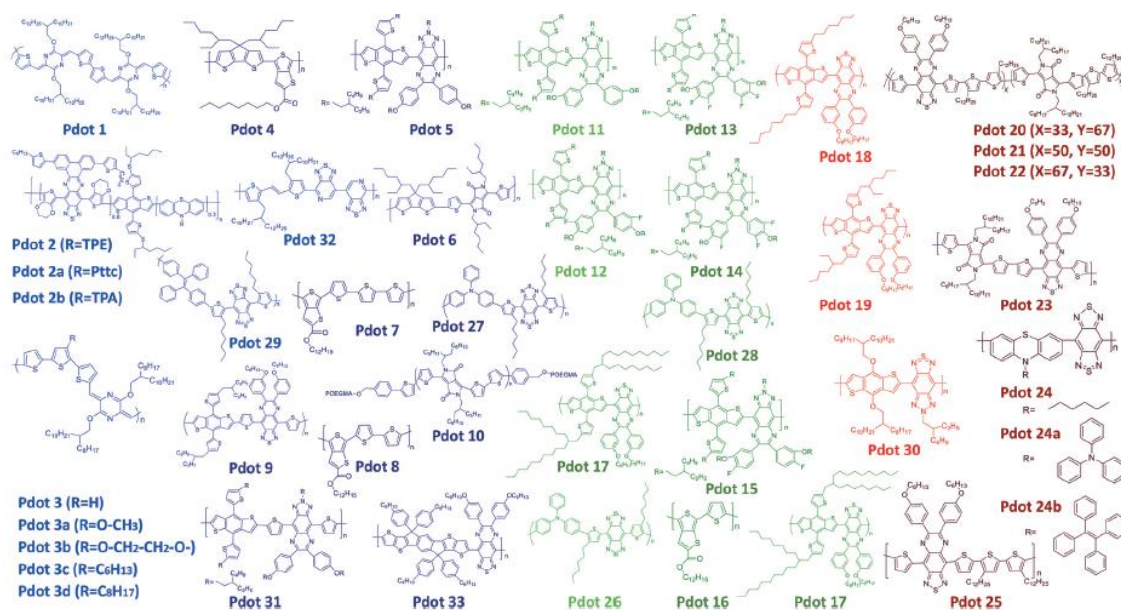


Figure 1.3. Chemical structures of D–A–D based conjugated polymers for Pdots.²⁷

This thesis focuses on the synthesis of D-A-D conjugated polymers using different polymerization methods. The three types of polymerization protocols were used oxidative polymerization, direct arylation polymerization, and stille polymerization. Oxidative polymerization is a method that can be employed to produce polymeric materials from a wide range of monomers. The monomers used in oxidative polymerization possess notable electron-donating properties and exhibit a significant tendency to oxidation. The basic characteristics of aromatic amines, phenols, thiophenols, and sulfur- and nitrogen-containing heterocycles are attributed to the presence of electron donor substituents in the heterocyclic ring. Oxidation of the monomer takes place when it is subjected to either an oxidizing agent or an applied voltage. During this process, the monomer molecules undergo the creation of cation or cation radical sites, which initiates the polymerization process (Figure 1.4). Oxidative polymerization is the chemical reaction in which covalent bonds are formed between monomer molecules by removing two protons. Different types of bonding occur between monomer units. There are three potential arrangements, namely "head-to-

head," "tail-to-tail," and "head-to-tail." Hence, oxidative polymerization leads to the creation of chains that display an extensive variety of monomer unit arrangements.⁴⁹

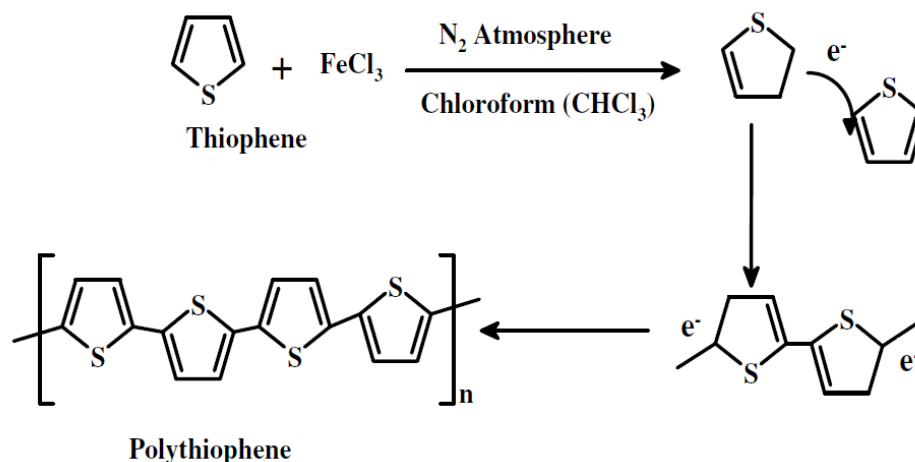


Figure 1.4. Mechanism of the typical oxidative polymerization.⁴⁹

Together with phosphine ligands and bases, palladium complexes can make it easier for electron-poor aromatic rings and aryl halides to couple up. This chemical process is also known as "direct arylation" (Figure 1.5). The procedure of synthesizing conjugated polymers has been performed utilizing direct arylation. In the past ten years, direct arylation (DAP) has become a new and innovative technique for producing conjugated polymers. Direct arylation is a method that does not require the use of an organometallic reagent, unlike traditional procedures. This instead allows a hydrogen atom (C-H) to be directly swapped out for an aryl halide in an aromatic hydrocarbon. Therefore, polymers can be successfully synthesized. This technique enables the quick and eco-friendly production of conjugated polymers. In addition, direct arylation can be used for copolymerization procedures.⁵⁰⁻⁵²

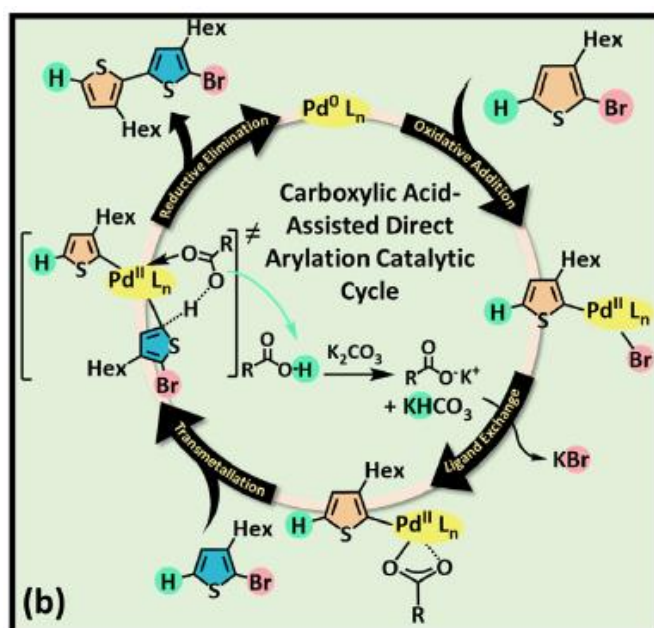


Figure 1.5. Mechanism of typical DAP reactions.⁵³

The Stille polymerization, which provides the basis for this polymerization process, involves the combination of organotin compounds with organic electrophiles in the presence of a palladium catalyst (Figure 1.6). Stille polymerization has drawn considerable interest due to its remarkable ability to generate polymers with semiconducting properties, which are essential for advancements in organic electronics, bioimaging, and biosensing.⁵⁴ Stille polymerization is highly desirable for the synthesis of polymers with diverse designs and functions, owing to its versatility and friendly reaction conditions. Furthermore, this approach's ability to withstand various functional groups enables the use of a wide range of monomers, broadening the potential applications of the resultant materials. The generalized Stille coupling catalytic cycle includes the palladium catalyst undergoing a transition between Pd(0) and Pd(ii), while the tin reagent transfers one coupling partner by transmetalation.⁵³

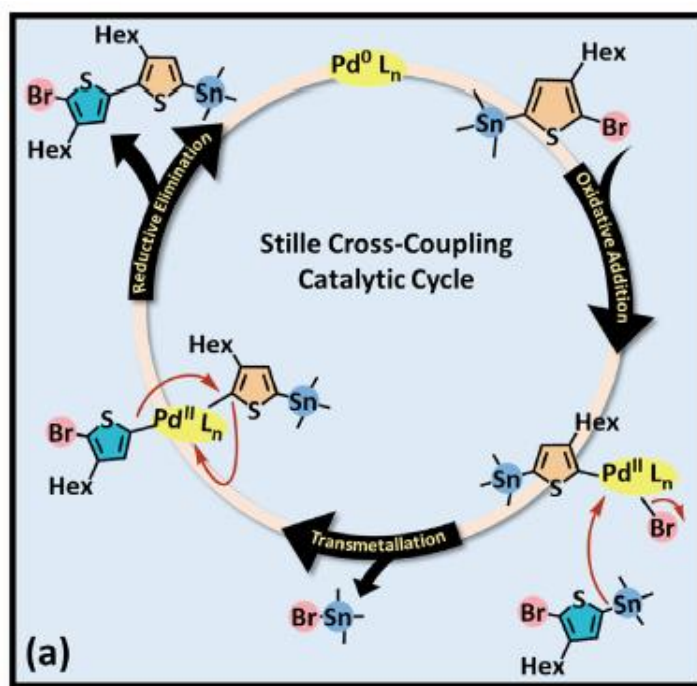


Figure 1.6. Mechanism of typical Stille polymerization.⁵³

1.3. Polymer dots

The recent studies, bioimaging of polymer nanoparticles or Pdots obtained from conjugated polymers^{55–57} showed high potential for diagnostic purposes^{58,59} and controlled drug delivery and treatment methods.¹⁸ Fluorescent Pdots allow long-term imaging due to their long π -conjugation in their main chain, thanks to their high fluorescence emission and colloidal durability. In addition to these features, the ability of polymer dots to penetrate the cell has been reported in recent years within the scope of in vivo and in vitro imaging studies.^{29,60}

Unlike conjugated polymer nanoparticles known to be larger than 30 nm in size, polymer dots (Pdot) having a size of 2–30 nm have higher absorption constant, high fluorescence quantum efficiency, photostability and colloidal stability^{42,60,61}. The basis of this improvement in optical properties is that the Pdots are composed of a single polymer chain and therefore the possibility of radiation-free decay is low. The difficulty of preparation methods and low reaction efficiency can be demonstrated as a disadvantage of Pdots in current studies. In 2019, article published by Özenler et al.²⁹, an easy-to-apply nanophase separation method is proposed to prepare a single chain

conjugated Pdot less than 5 nm in diameter. In this method, Pdots containing a rapid nanophase separation between water (weak solvent) and ethylene glycol (good solvent) and conjugated polyelectrolyte single chain are formed. Pdots have been found to have high absorbance coefficient, high quantum efficiency, brightness, photostability and colloidal stability. In addition, single-chain Pdots less than 10 nm in diameter can access crowded and hard-to-reach (restricted) intracellular areas and provide a more efficient opportunity to label these regions while providing significant advantages in penetrating into cellular compartments. Despite all these advances, Pdots' stimulation / radiation properties in the visible region can cause resolution restrictions in bioimaging and the cell or tissue must be exposed to high energy for a long time. In recent studies to solve these problems, NIR dyes have been proposed. In particular, the penetration of tissue deeper than visible light has made the use of NIR dyes in imaging biological samples. NIR fluorescent probes are of great importance, as they offer minimal interventional absorption, low biological autofluorescence, and high tissue penetration for biological applications in the NIR region.³⁶

Fluorescent polymer nanoparticles permit imaging without nonaggregation thanks to the colloidal stability and high fluorescence emission^{56,62}. Therefore, in vivo, and in vitro imaging studies reported the ability of penetration to cells in recent years.^{19,60} Moreover, with the unique properties of conjugated polymers which are high-emission and modifiable materials are used as a biomarker. In current studies, the difficulty of preparation methods and low reaction efficiency can be shown as the disadvantage of Pdots. The most common polymer nanoparticle and Pdot preparation methods known in the literature are nanoprecipitation⁶³ and miniemulsion.⁶⁴ Small-sized single-chain polymer dots can be prepared with modifications to commonly used protocols for example, the use of solid support⁶⁵ However, these known methods are disadvantageous in terms of the chemicals used and the length of the protocol. Polymer dot preparation methods in the literature:

- **Nanoprecipitation:** A stock solution is prepared by dissolving the conjugated polymer in a good solvent. The sample from the stock solution is added to the water and sonicated. The organic solvent is removed using nitrogen gas and the resulting solution is filtered through a microfilter (Figure 1.7).⁵⁷

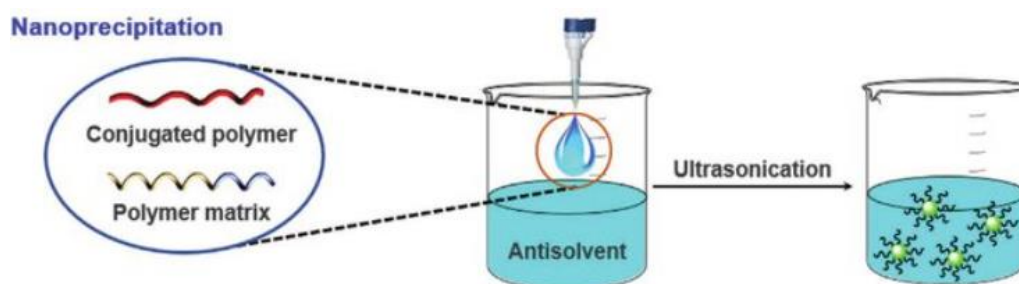


Figure 1.7. Nanoprecipitation method for preparation of Pdot.⁶⁶

- **Miniemulsion:** The conjugated polymer is dissolved in a water-immiscible organic solvent, for example, chloroform, and added to the surfactant-dissolved solution. To prepare the miniemulsion, sonicate the mixture for a specified duration. The emulsion solution is then stirred in a hot water bath to evaporate the organic solvent to obtain polymer dots (Figure 1.8).⁶⁴

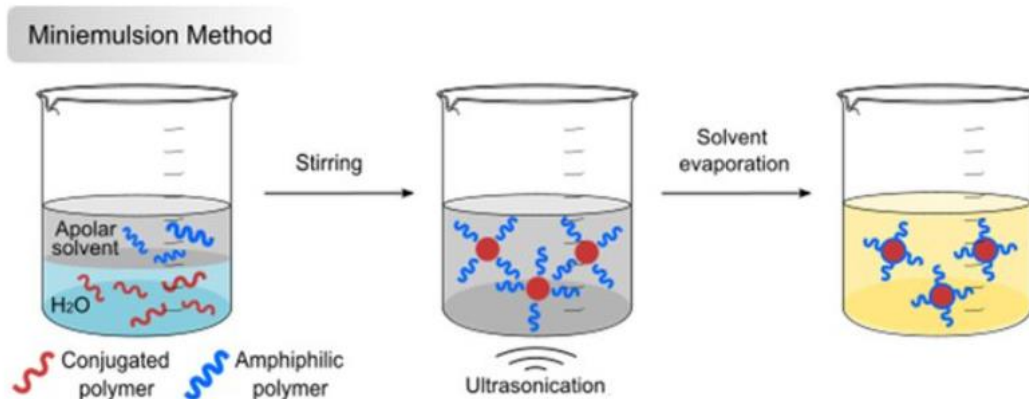


Figure 1.8. Miniemulsion method for preparation of Pdot.⁶⁷

- **Preparation of polymer dots using solid support:** Pdots that have been reprecipitated are introduced into silica particles that have been changed. The polymer dots are attached to the functional groups on the silica surface through a chemical reaction. They are then separated by centrifugation and purified by washing them with pure water. The polymer dot-silica complex is introduced into the aqueous phase while in a favorable solvent. Subsequently, the solvent is removed using nitrogen gas and heat. The final solution is achieved through sieving

using a membrane filter, and the polymer dots are separated from the silica surface using NaOH and Triton 100 (Figure 1.9).⁶⁵

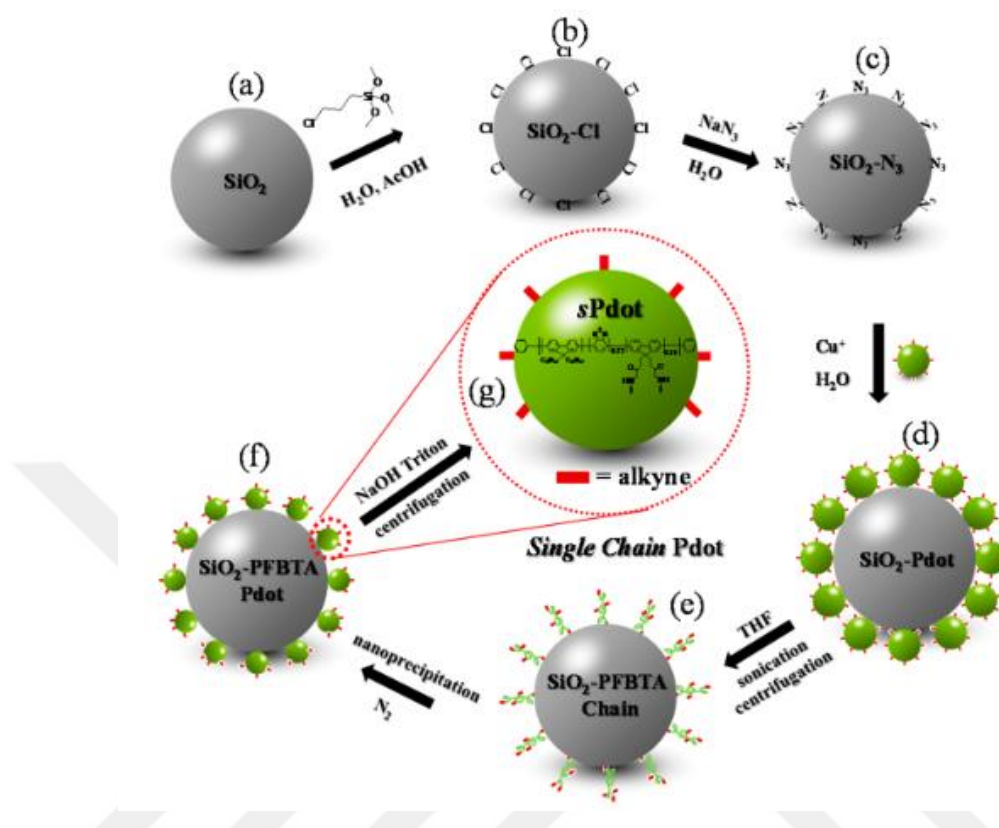


Figure 1.9. Preparation of polymer dots using solid support.⁶⁵

- **Nanophase separation:** As an alternative to known polymer dot preparation methods, Özenler et. al. have demonstrated for the first time that they can obtain conjugated polymer dots (Pdots) easily and without the use of toxic chemicals with the nanophase separation method. By the nanophase separation method, thiophene-based conjugated polyelectrolyte is dissolved in an organic good solvent and added to the water phase (poor solvent). In the final solution, single-chain polymer dots are obtained, surrounded by the good solvent but dispersed in the water phase (Figure 1.10).²⁹



Figure 1.10. Schematic illustration of the nanophase separation method for preparation of Pdot.²⁹

In summary, conjugated polymer-based nanoparticles with a diameter greater than 30 nm are converted into single-chain dots to obtain polymer dots (Pdot), and they have a high absorbance coefficient, photoluminescence, quantum yield, brightness, photostability, and colloidal stability. Thus, in biological applications, single-chain Pdots are expected to show distinct advantages over conjugated polythiophene nanoparticles. Additionally, single-chain Pdots with a diameter of less than 10 nm can get into crowded and difficult-to-reach (restricted) intracellular spaces, making it easier to label these areas.

1.4. Literature Works for NIR emissive Pdots

Conjugated polymers have been the subject of extensive research in recent decades for NIR imaging due to their outstanding advantages, including a greater extinction coefficient and better exciton migration compared to small fluorophore molecules. Consequently, many research groups globally have developed and created different polymer-based fluorophores specifically for fluorescent bioimaging purposes. Conjugated polymers are expected to be a strong candidate for the development of new fluorescent probes in bioimaging due to their improved photostability and image resolution, as well as their reduced cytotoxicity. The main problems encountered in the literature for NIR probes can be summarized as low quantum yield, low solubility in water, and low penetration into cells or tissues. The use of polymer dots with emission in the NIR spectral region as fluorescent probes, especially in vivo biological studies, has been an area of great interest.^{38,39,41}

Fluorescence emission in the NIR region is preferred because of its high tissue penetration and low autofluorescence.⁶⁸ In this part, some literature works were examined that have NIR emissive conjugated Pdots.

1. Rohatgi et al. prepared polymer dots with diameters in the range of 75–134 nm by nanoprecipitation method from conjugated polymer emitting in the spectral region of 800–1000 nm. With cell culture studies, it has been observed that polymer dots enter the cytoplasm of macrophage cells.⁶⁹
2. Compared to the frequently used polymer dots with visible region emission, materials with infrared emission reduce the limiting parameters such as self-absorption and scattering in the biological environment.⁷⁰
3. Liu et al. prepared polymer dots with an average size of 25 nm from quinoxaline-based conjugated polymers. The emission, colloidal stability, particle sizes, and tissue penetration of the prepared polymer dots in the 1000–1100 nm spectral region were characterized. In vivo experiments, it has been reported that tumor structures are visualized with high tissue penetration of polymer dots, fluorescence intensity, and signal-to-background ratio.⁷¹
4. In another in vivo study, polymer dots were obtained from the conjugated polymer with the help of a surfactant. The particle size distribution of the obtained polymer dots was found to be 60.3 ± 14.2 nm. The fluorescent polymer dots are characterized as having fluorescence emission at about 700 nm with a large Stokes shift and a quantum efficiency of 21%. In mouse modeling, cancer cells in lymph nodes were prominently observed thanks to the small particle sizes, high fluorescence properties, and good colloidal stability of the polymer dots. In line with these results, it is thought that the synthesized polymer dots have a high potential in the follow-up of a sentinel lymph node biopsy.⁴⁶
5. There are studies in literature to preserve the characteristic features of both structures with hybrid materials obtained from conjugated polymer dots and quantum dots.⁷² For example, Chiu and his group synthesized a hybrid material from the high fluorescence, photo-stability, and low toxicity of conjugated polymer Pdots and the high Stokes shift and narrow emission properties of quantum dots (QD) and used them in cell culture application. In 2-dimensional (2D) cell studies,

it has been reported that Pdot-QD nano-composite structures enter the cell and gather around the nucleus.⁷³

6. Wan et al. developed an organic nanofluorophore (p-FE) that glows in the NIR region for high-performance bioimaging. Because it gives off bright fluorescence at >1100 nm, p-FE allows blood flow monitoring in mouse brain vessels without touching them. p-FE can also do single-photon-based, three-dimensional (3D) confocal imaging of the blood vessels in fixed mouse brain tissue in layers as deep as 1.3 mm and with a high spatial resolution. Fluorescence imaging in the bi-color NIR region in vivo was also performed using p-FE as the vasculature imaging agent emitting in the 1100–1300 nm band and CNTs as the emitter above 1500 nm to illuminate the tumor in the mouse. However, it has been used in imaging applications by precipitating p-FE and PS-PEG copolymers; although this does not affect the fluorescent properties much, it makes it difficult to control the size of the Pdots and prevents the preparation of Pdots below 10 nm.⁷⁴
7. The synthesis of a NIR emissive polymer was achieved via a Suzuki coupling reaction in this study. Their polymer nanoparticles exhibited a quantum yield of 27%. The emission maxima was observed at 698 nm, resulting in a significantly large Stokes shift of around 233 nm in an aqueous solution. The nanoparticles included by DSPE-PEG-folic acid demonstrated enhanced thermal and photo stabilities as compared to commercially available quantum dots. Moreover, experiments conducted on a mouse model with hepatoma H22 tumors showed that the nanoparticles functioned as effective NIR fluorescent probes. They were able to identify tumors with a high level of contrast and specificity.⁷⁵
8. NIR emissive polymers were synthesized, and then they were used to produce polymer dots that can disperse in water. This was achieved using an ultrasonication-assisted co-precipitation approach. The absorption and emission maxima of the Pdots were seen at wavelengths of 380/710 nm and 450/720 nm, respectively. The NIR emissive Pdots exhibit a significant Stokes' shift of around 330 nm and have narrow emission bands with a complete width at half maximum of 44 nm. The quantum yield for the Pdots was measured to be 32% and 30%, respectively. Additionally, the use of single particle fluorescence imaging demonstrated that the synthesized NIR polymer dots exhibited a brightness that was more than three times greater than that of commercially available Qdot705.

Furthermore, these dots had a similar particle size when tested under the same conditions.⁷⁶

9. A set of highly bright NIR emitting polymer dots, based on quinoxaline, were developed. These Pdots exhibited a significant difference in the wavelengths at which they absorbed and emitted light. Specifically, the absorption maximum for the NIR polymer dots was 531 nm, while the emission maximum was at 695 nm. Similarly, the absorption maximum for another set of NIR polymer dots was at 529 nm, while the emission maximum was at 712 nm. The quantum yields of the Pdots in aqueous solutions were measured to be 9% and 8%, respectively.⁷⁷
10. Subsequently, by merely substituting the donor units, they successfully created polymer nanoparticles based on benzothiadiazole that exhibit emission ranging from bright green to NIR. The absorption and emission peaks for the other NIR emitting Pdots were seen at wavelengths of 505/720 nm and 602/758 nm, respectively. These characteristics make them very suitable for in vitro and in vivo NIR bioimaging applications, as they possess a long penetration depth, low autofluorescence, and minimal tissue scattering. The quantum yields of the recently produced Pdots in aqueous solutions were subsequently measured to be 10% and 2%, respectively. However, these values are quite low, which restricts their practical application.⁷⁸

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

4,8-Dibromobenzo[1,2-c:4,5-c']bis([1,2,5]thiadiazole) (BBT) (%95, ABCR), 3,4-Ethylenedioxythiophene (EDOT) (99%, Sigma Aldrich), Bis(triphenylphosphine)palladium(II) dichloride (Sigma Aldrich), Copper(I) bromide (Sigma Aldrich), 3,4-Dimethoxythiophene (%97, 3,4-Dimethoxythiophene), Tributyltin chloride (%96, Sigma Aldrich), n-Butyllithium solution (1.6 M in hexane), Triethylamine (99.5% Sigma Aldrich), N-Bromosuccinimide (%99, Sigma Aldrich), Dichloromethane (DCM), Chloroform, 3-Bromo-4-methylthiophene (95%, Sigma Aldrich), Copper(II) bromide (99%, Sigma Aldrich), Sodium methoxide (95%, powder, Sigma Aldrich), 1-Methyl-2-pyrrolidinone anhydrous, (99.5% Sigma Aldrich), Methanol (ACS reagent, $\geq 99.8\%$, Sigma Aldrich), Sodium Bromide (99%, Sigma Aldrich), Diethyl ether (99.5%, Sigma Aldrich), Magnesium sulfate (97%, Sigma Aldrich), Toluene (ACS reagent $\geq 99.7\%$, Sigma Aldrich), Silica gel (pore size 60 Å, 60-100 mesh, Merck), Hexane (95%, Sigma Aldrich), Ethyl acetate (99.7%, Sigma Aldrich), cetyltrimethylammoniumbromide (CTAB), Tetrahydrofuran (THF, ACS reagent, $\geq 99.9\%$, Sigma Aldrich), Iron(III)chloride (99.9%, Sigma Aldrich), Chloroform-d (99.8%, Merck), Hydrochloric acid (ACS reagent, $\geq 37\%$, Sigma Aldrich), Sodium hydroxide (ACS reagent, $\geq 97.0\%$, pellets), Sodium chloride (Sigma Aldrich), 1-Butanol (99%, Sigma Aldrich), were purchased. All substances were utilized in their original state without any further purification unless explicitly stated otherwise. For all studies, deionized water with a resistance of 18.2 MΩ·cm was used to reduce the influence of contaminants on the results.

2.2. Synthesis of donor-acceptor-donor based monomers

This section involves the synthesis of three distinct D-A-D monomers. They include the different donor sources as 3-Butoxy-4-methylthiophene (BuMT), 3,4-dibutoxythiophene (DiBuT), and 3,4-diethylenedioxythiophene (EDOT) with a 4,8-dibromobenzo[1,2-c:4,5-c']bis([1,2,5]thiadiazole) (BBT) as an acceptor unit, respectively monomer 1 (M1), monomer 2 (M2), and monomer 3 (M3). Therefore, the effect of the donor sources on the synthesis of NIR emissive polymers was examined.

2.2.1. Synthesis of Monomer 1

The synthesis information of M1 was given in Table 2.1. The synthesis of BuMT was performed as a source of donors for the M1. 1.18 grams of sodium metal were added to 20 mL of distilled 1-butanol. After 1 hour, a mixture of 25% by weight of sodium butoxide (NaOBu) in 1-butanol was prepared in an N₂ atmosphere as a dense, transparent color. In the next step, a round-bottom flask was filled with 1-methyl-2-pyrrolidone (NMP) (0.5 mL, 5.20 mmol) and sodium butoxide in butanol (NaOBu) (25% w/w, 10.0 mL, 33.0 mmol). After the mixture heated up to 130 °C, 3-bromo-4-methylthiophene (BMT) (1 g, 5.6 mmol) and CuBr (0.8 g, 5.6 mmol) were added to start the reaction. The reaction was carried out at 130 °C in the presence of N₂, using a condenser that was cooled with water. The reaction conditions were depicted in Figure 2.2. Subsequently, the mixture was allowed to cool to the ambient temperature, and 10 mL of a sodium bromide solution with a concentration of 0.25 g/mL was put in and rapidly mixed for 1 hour. Afterward, the mixture was subjected to three extractions using diethyl ether and subsequently separated. The organic phase, after being mixed, received a couple of washes with the aid of deionized water and was then subsequently dried using magnesium sulfate (MgSO₄). The solvent was later evaporated using a rotary evaporator and processed using column chromatography using hexane as a mobile phase. The retention factor (R_f) value was computed as 0.48 (mobile phase: hexane) in the TLC plate for the BuMT. The compound BuMT was synthesized as a light-yellow oil with a yield of 63% (0.60 g).

Table 2.1. Synthesis information for the M1.

Experiment	Components of Reactions	Quantity	Solvent	Temperature	Time
Preparation of NaOBu solution	Sodium metal	1.18 g (51 mmol)	No	Room Temperature	2 h
	1-Butanol	20 mL (218 mmol)			
Synthesis of BuMT	NaOBu (25% w/w)	10.0 mL (33.0 mmol)	Butanol	130 °C	72 h
	BMT	1 g, (5.6 mmol)			
	CuBr	0.8 g (5.6 mmol)			
	NMP	0.5 mL (5.2 mmol)			
Synthesis of Stannylated Precursor	BuMT	170 mg (1.0 mmol)	THF	-78°C	2 h
	n-butyl lithium (n-BuLi)	0.4 mL (1.0 mmol)		Room Temperature	Overnight
	tributyltin chloride	0.33 g (1.0 mmol)			
Synthesis of Monomer 1	stannylated precursor	(1.0 mmol)	Toluene	120°C	24 h
	BBT	115 mg (0.35 mmol)			
	(Pd(PPh ₃) ₂ Cl ₂)	78 mg (0.11 mmol)			

A D-A-D based M1, namely 4,8-di-3-butoxy-4 methylthiophene benzobisthiadiazole, was synthesized using a Stille coupling reaction. BuMT (1.0 mmol) was prepared under cryoreaction conditions (Figure 2.1). BuMT was dissolved in 10 mL of tetrahydrofuran (THF) and cooled to -78°C under an inert atmosphere. Following that, a solution of n-butyl lithium (n-BuLi) in 2.5 M hexane, measuring 0.4 mL (containing 1.0 mmol), was slowly added to the reaction mixture for 30 minutes. Following 1.5 hours of stirring at a temperature of -78°C, a solution of tributyltin chloride (0.33 g, 1.0 mmol) was slowly added for 10 minutes. After the completion of this process, the reaction medium was gradually brought up to room temperature and stirred for 8 hours under ambient conditions (Figure 2.2). Then, the resulting product was extracted using ethyl acetate, and water at least twice. After that, the organic phase was dried using MgSO₄, and then the solvent was evaporated by a rotary evaporator (The R_f value was calculated as 0.98 (mobile phase: 1. hexane/triethylamine (TEA) 10:1 for neutralizing the TLC plate, 2. hexane/DCM 1:1) in the TLC plate for the one-sided stannylated BuMT).

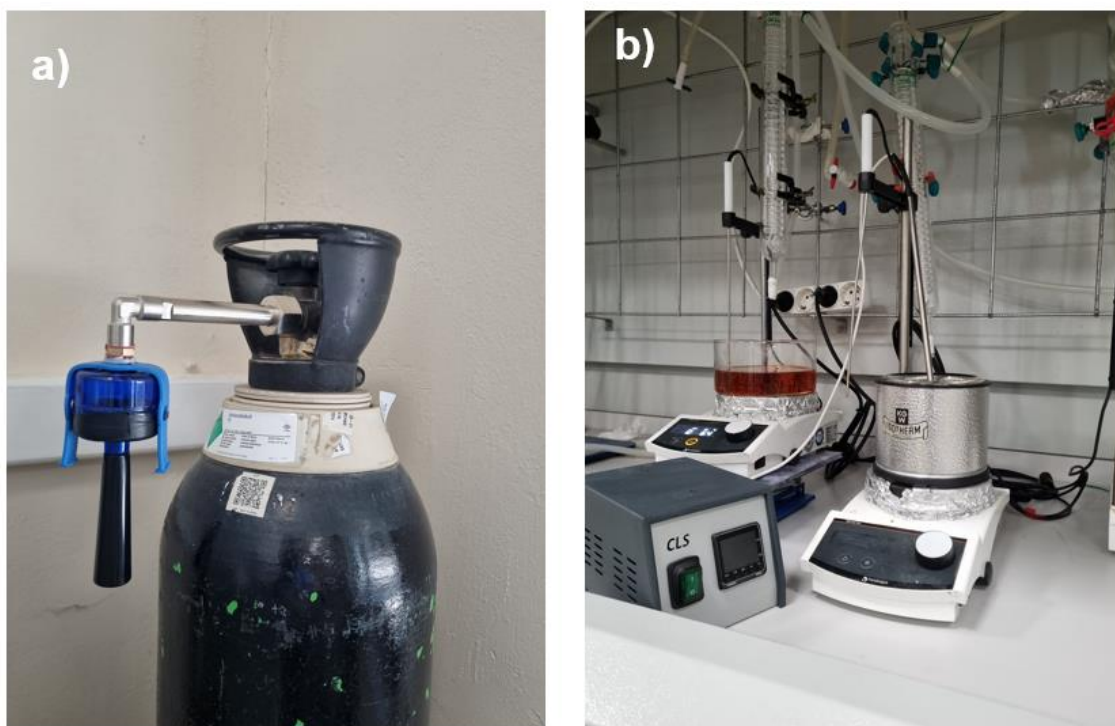


Figure 2.1. Photograph of the a) dry ice machine, and b) reaction medium that is controlled by the thermometer.

Then, the stannylated precursor (1.0 mmol) and BBT (115 mg, 0.35 mmol) were dissolved in 10 mL of toluene. In the next step, bis(triphenylphosphine)palladium chloride ($\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$) (78 mg) was added to the mixture as a catalyst. The solution was stirred at a temperature of 120°C in the presence of N_2 and a condenser cooled with water for 24 hours (Figure 2.2). After the reaction was finished, it was cooled to room temperature, and extracted using ethyl acetate. The resulting mixture was then washed with water at least two times. Afterward, the solvent of the substance was dried using MgSO_4 , and then the solvent was removed using a vacuum. Subsequently, the product proceeded to be purified using column chromatography using a hexane/dichloromethane (DCM) eluent in a ratio of 1:1. The R_f value was calculated as 0.45 (mobile phase: hexane/DCM 1:1) in the TLC plate for the monomer 1. It was then stored at a temperature of $+4^\circ\text{C}$, making it readily available for usage. The monomer was obtained in the form of a dark blue substance, weighing 90 mg and with a yield of 51%.

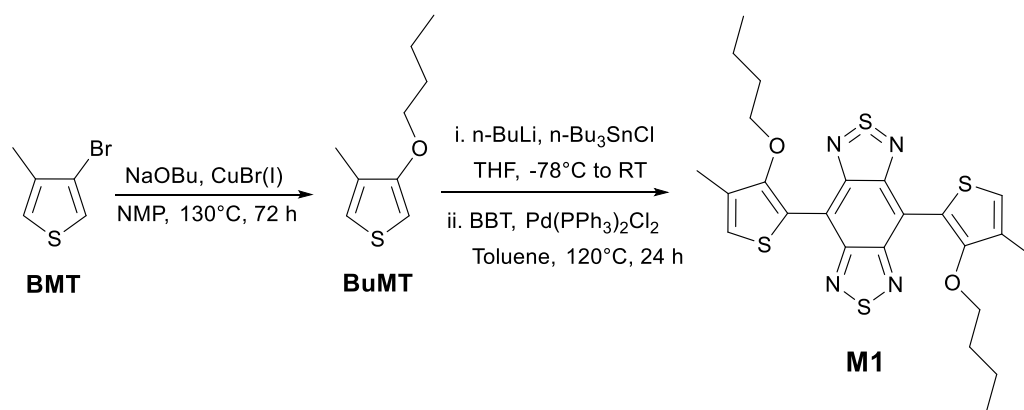


Figure 2.2. Synthesis protocol for M1.

2.2.2. Synthesis of Monomer 2

The synthesis information of M2 was given in Table 2.2. The synthesis of DiBuT was carried out as a donor source for M2 (4,8-di-3,4-dibutoxythiophenebenzobisthiadiazole). Firstly, a mixture of 3,4-dimethoxythiophene (DMT) (0.5 g, 3.47 mmol), 1-butanol (1.37 mL, 15 mmol), and NaHSO₄ (112 mg, 0.94 mmol) in 10 mL of toluene was added to a round bottom flask. This reaction mixture was heated at 100 °C for 24 hours under a N₂ atmosphere (Figure 2.3). After 24 hours, the reaction mixture was allowed to cool to room temperature. Toluene was evaporated to concentrate the reaction solution by a rotary evaporator, and the remaining reaction mixture was extracted three times with diethyl ether, subsequently washed with water, and dried over magnesium sulfate. Then, diethyl ether was evaporated by a rotary evaporator. The crude product was purified with column chromatography (silica gel, hexane:DCM 1:1) to give the DiBuT a yellowish oil with a yield of 63% (0.50 g). The R_f value was computed as 0.83 (mobile phase: hexane/DCM 1:1) in the TLC plate for the DiBuT.

A solution of DiBuT (1.0 mmol) was dissolved with dried THF (7 mL) in an N₂ atmosphere. Then, it was cooled to -78°C, and n-BuLi (in 1.6 M hexane, 1 mmol) was added drop by drop for 30 minutes. After stirring for a total of 1.5 hours at this temperature, tributyltin chloride (n-Bu₃SnCl) (1 mmol) was slowly added to the reaction medium (Figure 2.3). At the end of this process, the reaction is slowly warmed to room temperature and stirred overnight. Then, it was extracted with DCM and dried with

MgSO₄, and it was evaporated. The residue was purified by column chromatography with hexane-ethylacetate (20:1). Before starting column chromatography, silica gel was washed with triethylamine-hexane (1:10). In addition, TLC analysis was performed with triethylamine-hexane (1:10) to neutralize the TLC plate, and then hexane-ethylacetate (20:1) was used as an eluent. The R_f value was computed as 0.75 for the one-sided stannylated DiBuT. After the one-sided stannylated DiBuT (tributyl(3,4-dibutoxythiophen-2-yl)stannane) was obtained, it was used in the next step. In the synthesis of D-A-D monomer stage, the reaction was set with a mixture of the one-sided stannylated compound (73 mg, 0.14 mmol), BBT (19.7 mg, 0.056 mmol), and Pd(PPh₃)₂Cl₂ (%5) in toluene (5 mL) under N₂ atmosphere. The mixture is stirred at 120°C for 24 hours. (Figure 2.3). After cooling to room temperature, the reaction mixture was precipitated with methanol. The R_f value was calculated as 0.45 (mobile phase: hexane/ethylacetate 20:1) in the TLC plate for the monomer 2. The M2 was obtained with a yield of 55% as a dark blue color.

Table 2.2. Synthesis information for the M2.

Experiment	Components of Reactions	Quantity	Solvent	Temperature	Time
Synthesis of DiBuT	1-Butanol	1.37 mL (15 mmol)	Toluene	100 °C	24 h
	DMT	0.5 g (3.47 mmol)			
	NaHSO ₄	112 mg (0.94 mmol)			
Synthesis of Stannylated Precursor	DiBuT	228 mg (1.0 mmol)	THF	-78°C	2 h
	n-butyl lithium (n-BuLi)	0.4 mL (1.0 mmol)		Room Temperature	Overnight
	tributyltin chloride	0.33 g (1.0 mmol)			
Synthesis of Monomer 2	stannylated precursor	73 mg, 0.14 mmol	Toluene	120°C	24 h
	BBT	19.7 mg, 0.056 mmol			
	(Pd(PPh ₃) ₂ Cl ₂)	(0.007 mmol)			

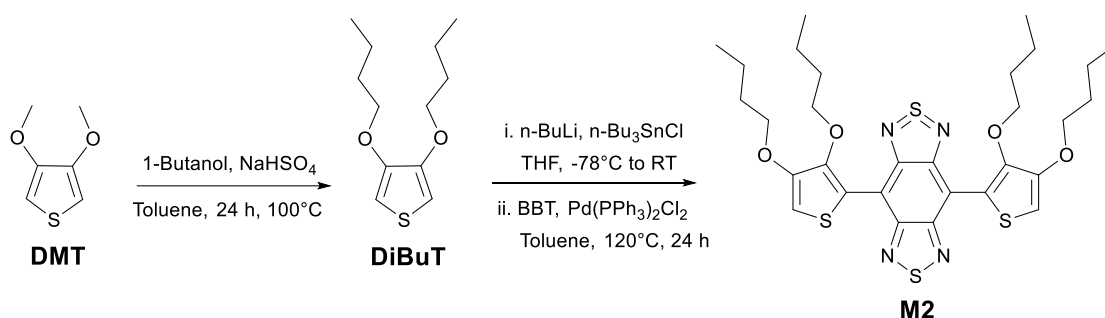


Figure 2.3. Synthesis protocol for M2.

2.2.3. Synthesis of Monomer 3

The synthesis information of M3 was given in Table 2.3. A solution of EDOT (1.0 mmol) was dissolved with dried THF (7 mL) in an N₂ atmosphere. Then, it was cooled to -78°C, and n-BuLi (in 1.6 M hexane, 1 mmol) was added drop by drop for 30 minutes. After stirring for a total of 1.5 hours at this temperature, n-Bu₃SnCl (1 mmol) was slowly added to the reaction medium (Figure 2.4). At the end of this process, the reaction is slowly warmed to room temperature and stirred overnight. Then, it was extracted with DCM and dried with MgSO₄, and it was evaporated. The residue was purified by column chromatography with hexane-ethylacetate (20:1). Before starting column chromatography, silica gel was washed with triethylamine-hexane (1:10). In addition, TLC analysis was performed with triethylamine-hexane (1:10) to neutralize the TLC plate, and then hexane-ethylacetate (20:1) was used as an eluent. The R_f value was calculated as 0.65 for the one-sided stannylated EDOT. After the one-sided stannylated EDOT (tributyl(2,3-dihydrothieno[3,4-b][1,4]dioxin-5-yl)stannane) was obtained, it was used in the next step. In the synthesis of D-A-D monomer stage, the reaction was set with a mixture of the one-sided stannylated compound (73 mg, 0.14 mmol), BBT (19.7 mg, 0.056 mmol), and Pd(PPh₃)₂Cl₂ (%5) in toluene (5 mL) under N₂ atmosphere. The mixture is stirred at 120°C for 24 hours. (Figure 2.4). After cooling to room temperature, the reaction mixture was precipitated with methanol. The R_f value was evaluated as 0.08 (mobile phase: hexane/ethylacetate 20:1) in the TLC plate for the monomer 3. The M3 (4,8-di-3,4-ethylenedioxythiophenebenzobisthiadiazole) was obtained with a yield of 60 % as a dark blue color.

Table 2.3. Synthesis information for the M3.

Experiment	Components of Reactions	Quantity	Solvent	Temperature	Time
Synthesis of Stannylated Precursor	EDOT	(1.0 mmol)	THF	-78°C	2 h
	n-butyl lithium (n-BuLi)	0.4 mL (1.0 mmol)		Room Temperature	Overnight
	tributyltin chloride	0.33 g (1.0 mmol)			
Synthesis of Monomer 3	stannylated precursor	73 mg, 0.14 mmol	Toluene	120°C	24 h
	BBT	19.7 mg, 0.056 mmol			
	(Pd(PPh ₃) ₂ Cl ₂)	(0.007 mmol)			

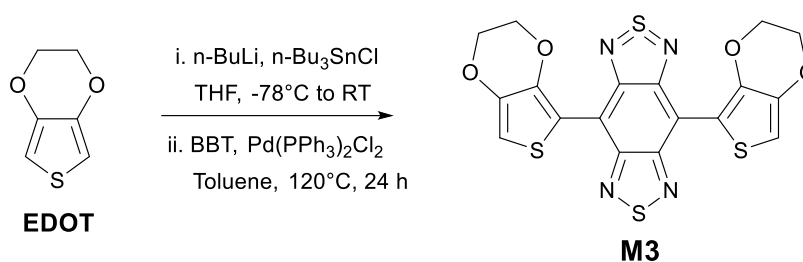


Figure 2.4. Synthesis protocol for M3.

2.3. Synthesis of NIR Emissive Conjugated Polymers

NIR emissive conjugated polymers were synthesized with various polymerization protocols. These are oxidative polymerization, direct arylation polymerization, and stille polymerization.

2.3.1. Oxidative Polymerization

The synthesis informations of oxidative polymerization products were given in Table 2.4. The oxidative polymerization of M1 was carried out to yield a nonionic polymer that emits near-infrared light. In Figure 2.5, a solution containing 8.4 mg (0.016 mmol) of M1 in 2 mL of chloroform was slowly added to a solution of anhydrous FeCl_3 (15 mg, 0.09 mmol) in 2 mL of chloroform. The mixture was then stirred for 48 hours at room temperature under a nitrogen atmosphere. After two days, the solvent was evaporated and concentrated, and the remaining substance was washed with methanol (four times). Subsequently, the polymer product (P1) received a drying process within a desiccator.

Table 2.4. Synthesis information for the oxidative polymerization products.

Experiment	Components of Reactions	Quantity	Solvent	Temperature	Time
Polymerization for P1	Monomer 1	8.4 mg (0.016 mmol)	Chloroform	Room Temperature	48 h
	FeCl_3	15 mg (0.09 mmol)			
Polymerization for P2	Monomer 2	16 mg (0.025 mmol)	Chloroform	Room Temperature	48 h
	FeCl_3	17 mg (0.1 mmol)			
Polymerization for P3	Monomer 3	10 mg (0.021 mmol)	Chloroform	Room Temperature	48 h
	FeCl_3	13.6 mg (0.084 mmol)			

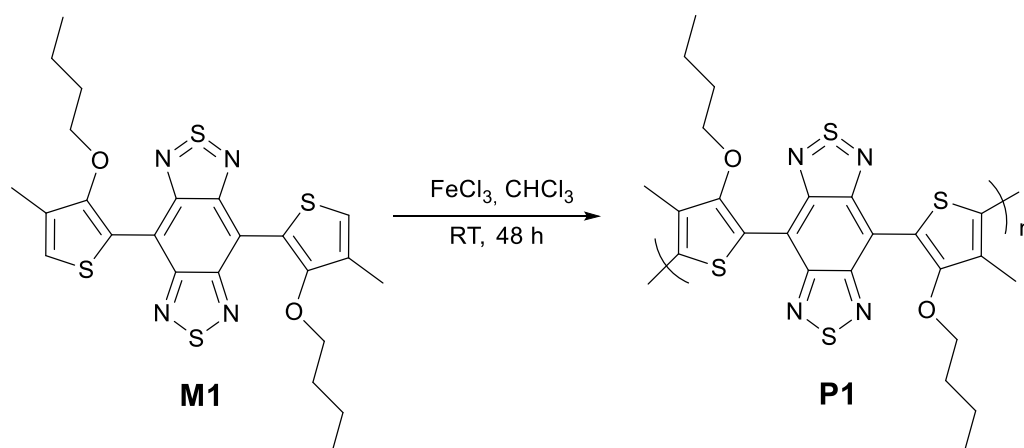


Figure 2.5. Oxidative polymerization protocol for P1.

The oxidative polymerization of M2 was performed to yield a NIR emissive polymer. In Figure 2.6, a solution containing 16 mg (0.025 mmol) of M2 in 2 mL of chloroform was slowly added to a solution of anhydrous FeCl_3 (17 mg, 0.1 mmol) in 2 mL of chloroform. The mixture was then stirred for 48 hours at room temperature under a nitrogen atmosphere. After two days, the solvent was evaporated and concentrated, and the remaining substance was washed with methanol (four times). Subsequently, the polymer product (P2) received a drying process within a desiccator.

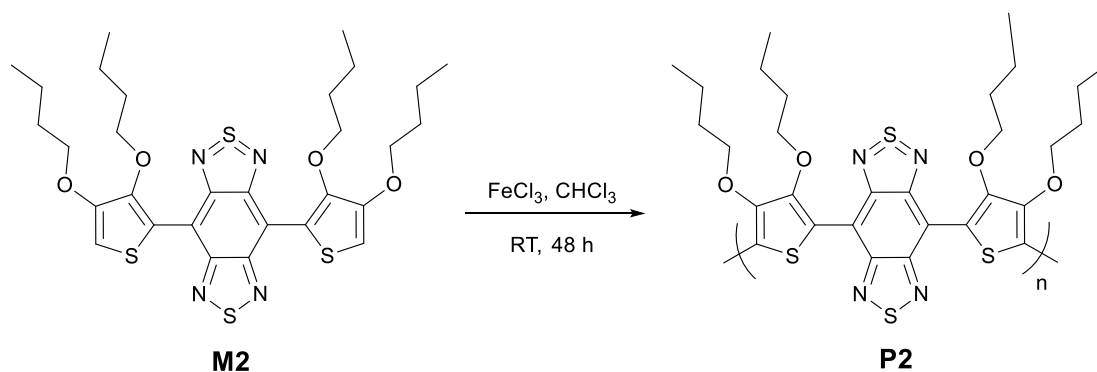


Figure 2.6. Oxidative polymerization protocol for P2.

The oxidative polymerization of M3 was carried out to yield a NIR emissive polymer. In Figure 2.7, a solution containing 10 mg (0.021 mmol) of M3 in 2 mL of chloroform was slowly added to a solution of anhydrous FeCl_3 (13.6 mg, 0.084 mmol) in 2 mL of chloroform. The mixture was then stirred for 48 hours at room temperature under a nitrogen atmosphere. After two days, the solvent was evaporated and

Table 2.5. Synthesis information for the DArP trials.

Experiment	Components of Reactions	Quantity	Solvent	Temperature	Time
Synthesis of Br-BuMT	N-Bromosuccinimide (NBS)	52.3 mg (0.294 mmol)	DCM	0 °C	2 h
	BuMT	50 mg (0.294 mmol)			
DArP-1 for PBUMT	Br-BuMT	7.4 mg (0.03 mmol)	NMP	80 °C	48 h
	K ₂ CO ₃	10.4 mg (0.075 mmol)			
	Pd(OAc) ₂	0.67 mg (0.003 mmol)			
	PivOH	0.92 mg (0.009 mmol)			
DArP-2 for PBUMT	Br-BuMT	7.8 mg (0.031 mmol)	DMF	80 °C	48 h
	K ₂ CO ₃	10.7 mg (0.0775 mmol)			
	Pd(OAc) ₂	0.35 mg (0.0015 mmol)			
	TBAB	10 mg (0.031 mmol)			
DArP-3 for PBUMT	Br-BuMT	9.8 mg (0.039 mmol)	DMF	80 °C	48 h
	KOAc	8.8 mg (0.09 mmol)			
	Pd(OAc) ₂	0.44 mg (0.00195 mmol)			
	TBAB	12.6 mg (0.039 mmol)			
DArP-4 for P4	BuMT	9.7 mg (0.057 mmol)	NMP	80 °C	48 h
	BBT	20 mg (0.057 mmol)			
	K ₂ CO ₃	19.8 mg (0.143 mmol)			
	Pd(OAc) ₂	1.3 mg (0.0057 mmol)			
	PivOH	1.8 mg (0.0173 mmol)			
DArP-5 for P4	BuMT	9.9 mg (0.058 mmol)	DMF	80 °C	48 h
	BBT	20.4 mg (0.058 mmol)			
	K ₂ CO ₃	20 mg (0.145 mmol)			
	Pd(OAc) ₂	0.65 mg (0.00265 mmol)			
	TBAB	18.7 mg (0.058 mmol)			
DArP-6 for P4	BuMT	9.7 mg (0.057 mmol)	DMF	80 °C	48 h
	BBT	20 mg (0.057 mmol)			
	KOAc	14.2 mg (0.145 mmol)			
	Pd(OAc) ₂	0.65 mg (0.00265 mmol)			
	TBAB	18.4 mg (0.058 mmol)			
DArP for P5	DiBuT	25 mg (0.109 mmol)	NMP	80 °C	48 h
	BBT	38.5 mg (0.109 mmol)			
	K ₂ CO ₃	38 mg (0.273 mmol)			
	Pd(OAc) ₂	2.5 mg (0.0109 mmol)			
	PivOH	3.4 mg (0.033 mmol)			

To test the direct arylation polymerization, three different DArP protocols were selected to synthesize poly BuMT (PBuMT) (Figure 2.8). Details of the synthesis protocol were summarized for DArP-1, DArP-2, and DArP-3, respectively. For DArP-1, Br-BuMT (7.4 mg, 0.03 mmol), potassium carbonate (K₂CO₃) as a base (10.4 mg, 0.075 mmol), palladium(II) acetate (Pd(OAc)₂) as a catalyst (0.67 mg, 0.003 mmol), and pivalic acid (PivOH) as a carboxylate ligand (0.92 mg, 0.009 mmol) were dissolved in 1 mL of NMP in a test tube. DArP-2, Br-BUMT (7.8 mg, 0.031 mmol), K₂CO₃ (10.7 mg, 0.0775 mmol), Pd(OAc)₂ (0.35 mg, 0.0015 mmol), and tetrabutylammonium bromide (TBAB) as a phase transfer agent (10 mg, 0.031 mmol) were dissolved in 1 mL of DMF in a test tube. DArP-3, Br-BUMT (9.8 mg, 0.039 mmol), potassium acetate (KOAc) (8.8 mg, 0.09 mmol), Pd(OAc)₂ (0.44 mg, 0.00195 mmol), and TBAB (12.6 mg, 0.039 mmol) were dissolved in 1 mL of DMF in a test tube. The reaction mixture were purged with nitrogen gas for 5 minutes, and then reactions were carried out for 48

hours at 80°C. After 2 days, the products were precipitated in methanol and dried in the desiccator. PBuMT was obtained with three different DArP protocols.

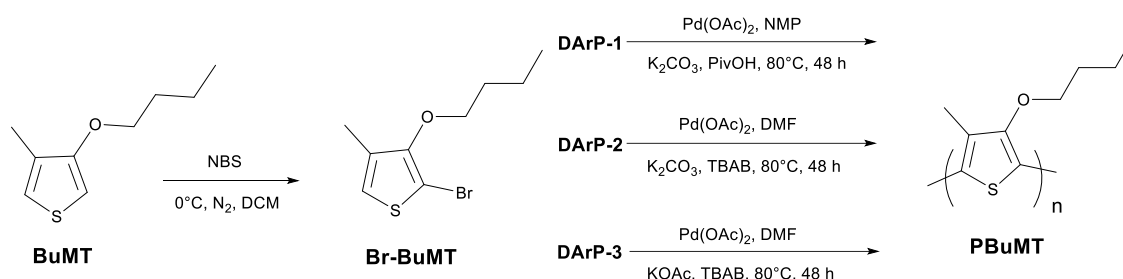


Figure 2.8. DArP protocols for PBuMT.

After the testing of DArP protocols to synthesize PBuMT, the same three DArP protocols were used to synthesize poly BuMT-BBT (P4) (Figure 2.9). Details of the synthesis protocols were summarized for DArP-4, DArP-5, and DArP-6, respectively. For DArP-4; BuMT (9.7 mg, 0.057 mmol), BBT (20 mg, 0.057 mmol), K₂CO₃ (19.8 mg, 0.143 mmol), Pd(OAc)₂ (1.3 mg, 0.0057 mmol), and PivOH (1.8 mg, 0.0173 mmol) were dissolved in 1 mL of NMP in a test tube. For DArP-5, BuMT (9.9 mg, 0.058 mmol), BBT (20.4 mg, 0.058 mmol), K₂CO₃ (20 mg, 0.145 mmol), Pd(OAc)₂ (0.65 mg, 0.00265 mmol), and TBAB (18.7 mg, 0.058 mmol) were dissolved in 1 mL of DMF in a test tube. For DArP-6, BuMT (9.7 mg, 0.057 mmol), BBT (20 mg, 0.057 mmol), KOAc (14.2 mg, 0.145 mmol), Pd(OAc)₂ (0.65 mg, 0.00265 mmol), and TBAB (18.4 mg, 0.058 mmol) were dissolved in 1 mL of DMF in a test tube. The solutions were purged with nitrogen gas for 5 minutes, and then reactions were carried out for 48 hours at 80°C. After 2 days, the products were precipitated in methanol and dried in a desiccator.

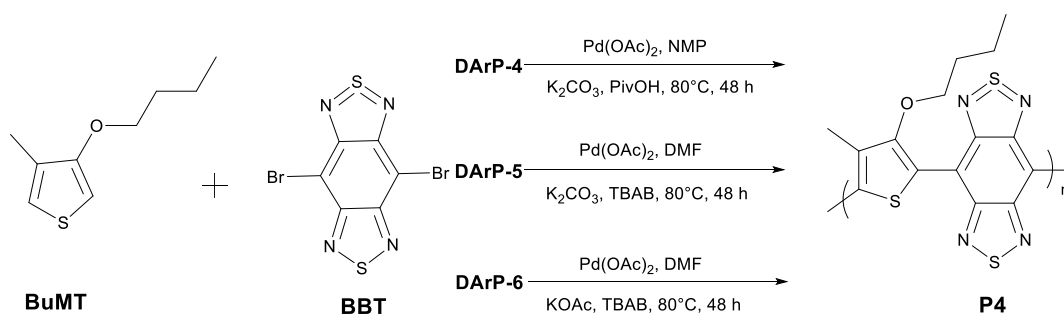


Figure 2.9. DArP protocols for P4.

To increase the polymeric product scale via the DArP protocol, DiBuT was used as a donor source instead of BuMT. Therefore, poly DiBuT-BBT (P5) was aimed to synthesize (Figure 2.10). In this reaction, DiBuT (25 mg, 0.109 mmol), BBT (38.5 mg, 0.109 mmol), K_2CO_3 (38 mg, 0.273 mmol), $Pd(OAc)_2$ (2.5 mg, 0.0109 mmol), and PivOH (3.4 mg, 0.033 mmol) were dissolved in 2 mL of NMP in a schlenk tube. The reaction solution was purged with nitrogen gas for 5 minutes, and then reactions were carried out for 48 hours at 80°C. After 2 days, the products were precipitated in methanol and dried in a desiccator.

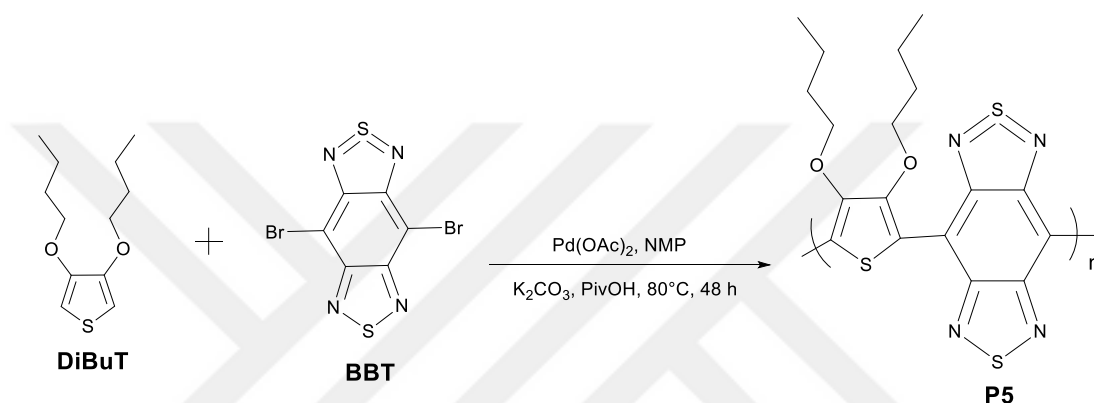


Figure 2.10. DArP protocol for P5.

2.3.3. Stille Polymerization

The synthesis informations of Stille polymerization products were given in Table 2.6. Stille polymerization, two kinds of donor compounds were used: DiBuT and EDOT, with an acceptor unit of BBT. Before polymerization, the synthesis of two-sided stannylated donor compounds was carried out. Accordingly, a solution of DiBuT (200 mg, 0.88 mmol) was dissolved in 7 mL of N_2 atmosphere, and then it was cooled to -78°C. Afterward, *n*-BuLi (in 1.6 M hexane, 2.2 mL, 3.52 mmol) solution was added drop by drop to the DiBuT solution for 30 minutes. Then, the reaction mixture was stirred for 1 hour at room temperature. After stirring for 1.5 hours, the reaction mixture was cooled to -78°C to add the *n*- Bu_3SnCl (0.96 mL, 3.52 mmol). At the end of this process, the reaction was slowly warmed to room temperature and stirred overnight (Figure 2.11). After the completion of the reaction, it was extracted with DCM and

dried with MgSO_4 . Then it was filtrated, and it was evaporated. Then, the two-sided stannylated DiBuT ((3,4-dibutoxythiophene-2,5-diyl)bis(tributylstannane)) residue was purified by column chromatography with hexane as an eluent. Before the column chromatography, silica gel was washed with a triethylamine-hexane (1:10) solvent mixture. The R_f value was evaluated as 0.88 for the two-sided stannylated DiBuT. Therefore, the column chromatography purification process was successfully performed for two-sided stannylated DiBuT.

Table 2.6. Synthesis information for the Stille polymerization products.

Experiment	Components of Reactions	Quantity	Solvent	Temperature	Time
Synthesis of Stannylated EDOT	EDOT	200 mg, 1.42 mmol	THF	-78°C	2 h
	n-butyl lithium (n-BuLi)	3.53 mL, 5.64 mmol		Room Temperature	Overnight
	tributyltin chloride	1.53 mL, 5.64 mmol			
Synthesis of Stannylated DiBuT	DiBuT	200 mg, 0.88 mmol	THF	-78°C	2 h
	n-butyl lithium (n-BuLi)	2.2 mL, 3.52 mmol		Room Temperature	Overnight
	tributyltin chloride	0.96 mL, 3.52 mmol			
Polymerization for P6	stannylated precursor EDOT	0.084 mmol	Toluene	120°C	24 h
	BBT	0.084 mmol			
	(Pd(PPh ₃) ₂ Cl ₂)	0.0084 mmol			
Polymerization for P7	stannylated precursor EDOT	0.063 mmol	Toluene	120°C	24 h
	stannylated precursor DiBuT	0.021 mmol			
	BBT	0.084 mmol			
	(Pd(PPh ₃) ₂ Cl ₂)	0.0084 mmol			
Polymerization for P8	stannylated precursor EDOT	0.042 mmol	Toluene	120°C	24 h
	stannylated precursor DiBuT	0.042 mmol			
	BBT	0.084 mmol			
	(Pd(PPh ₃) ₂ Cl ₂)	0.0084 mmol			
Polymerization for P9	stannylated precursor EDOT	0.021 mmol	Toluene	120°C	24 h
	stannylated precursor DiBuT	0.063 mmol			
	BBT	0.084 mmol			
	(Pd(PPh ₃) ₂ Cl ₂)	0.0084 mmol			
Polymerization for P10	stannylated precursor DiBuT	0.084 mmol	Toluene	120°C	24 h
	BBT	0.084 mmol			
	(Pd(PPh ₃) ₂ Cl ₂)	0.0084 mmol			

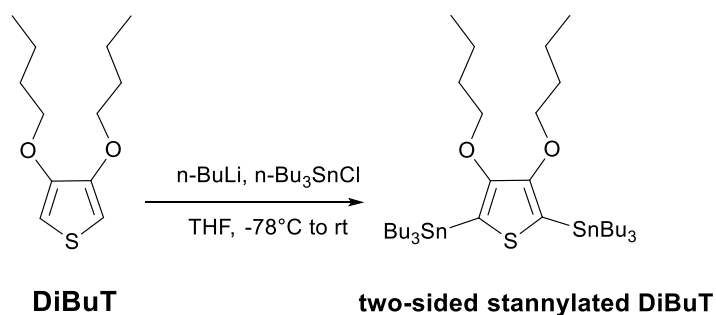


Figure 2.11. Synthesis protocol for two-sided stannylated DiBuT.

To obtain two-sided stannylated EDOT, a solution of EDOT (200 mg, 1.42 mmol) was dissolved in 7 mL of N_2 atmosphere, and then it was cooled to -78°C .

Afterward, n-BuLi (in 1.6 M hexane, 3.53 mL, 5.64 mmol) solution was added drop by drop to the EDOT solution for 30 minutes. Then, the reaction mixture was stirred for 1 hour at room temperature. After stirring for 1.5 hours, the reaction mixture was cooled to -78°C to add the n-Bu₃SnCl (1.53 mL, 5.64 mmol). At the end of this process, the reaction was slowly warmed to room temperature and stirred overnight (Figure 2.12). After the completion of the reaction, it was extracted with DCM and dried with MgSO₄. Then it was filtrated, and it was evaporated. Then, the two-sided stannylated EDOT (5,7-bis(tributylstannyl)-2,3-dihydrothieno[3,4-b][1,4]dioxine) residue was purified by column chromatography with hexane as an eluent. Before the column chromatography, silica gel was washed with a triethylamine-hexane (1:10) solvent mixture. The R_f value was calculated as 0.88 for the two-sided stannylated EDOT. Therefore, the column chromatography purification process was successfully performed for two-sided stannylated EDOT.

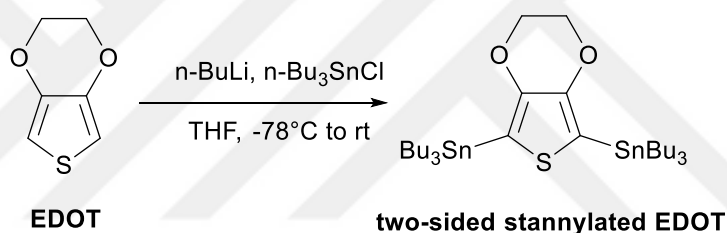


Figure 2.12. Synthesis protocol for two-sided stannylated EDOT.

After obtaining the two-sided stannylated donor compounds, polymerization reaction series were performed to synthesize five NIR emissive polymers (P6–10) via Stille polymerization. These NIR emissive polymers were prepared with a different donor mol ratio, as seen in the Table 2.6. Among the synthesized NIR emissive polymers, P6 and P10 were synthesized as homopolymers. Also, other polymers (P7–8–9) were prepared as copolymers using the two types of donors. Accordingly, a typical procedure was carried out: a mixture of the two-sided stannylated EDOT, BBT, two-sided stannylated DiBuT, and Pd(PPh₃)₂Cl₂ (%10) in toluene (5 mL) was added to a round bottom flask and stirred at 120°C for 24 hours under N₂ gas (Figure 2.13). After 24 hours, the reaction mixture was cooled to room temperature, and then the mixture was precipitated with methanol. The precipitants were dried in a desiccator. At the end of the procedure, five different NIR emissive polymers were successfully obtained.

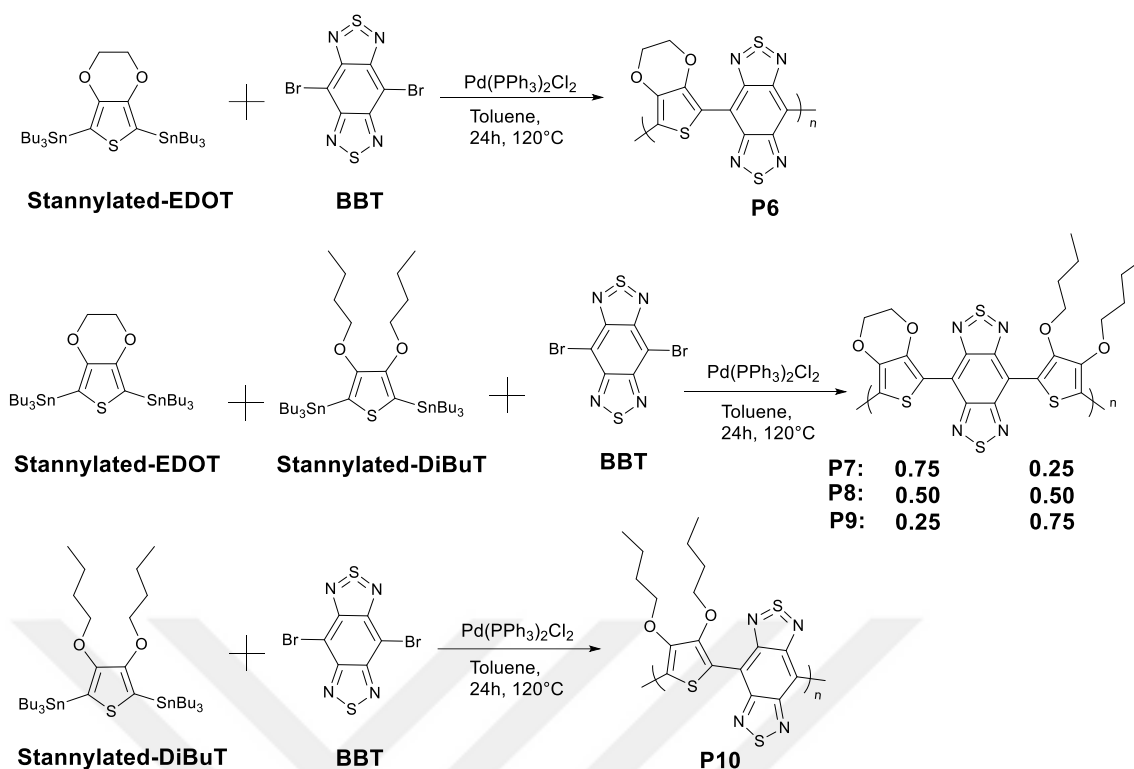


Figure 2.13. Stille polymerization protocols for P6-10 (P7-8-9 NIR emissive polymer structure were given with content ratios of the donor compounds).

2.4. Preparation of the Pdots

NIR emissive Pdots were prepared via the miniemulsification method. The photograph of an ultrasonicator instrument that was used to obtain Pdots is shown in Figure 2.14. In this step, only the P1 NIR emissive polymer was used as a model polymer in its application areas. The miniemulsification process was shown in Figure 2.15, illustrating how to produce Pdots from P1 (1 mg) dissolved in a water-immiscible organic solvent chloroform (1 mL, CHCl_3) with the addition of cetyltrimethylammoniumbromide (CTAB) as a surfactant solution (84 mg surfactant in 10 mL Milli-Q water). After applying the mixture to 250-watt sonication for two minutes, a cloudy brownish solution was obtained. The resulting sample was subsequently stirred in a hot water bath to remove the organic solvent through evaporation.



Figure 2.14. Photograph of the ultrasonicator.

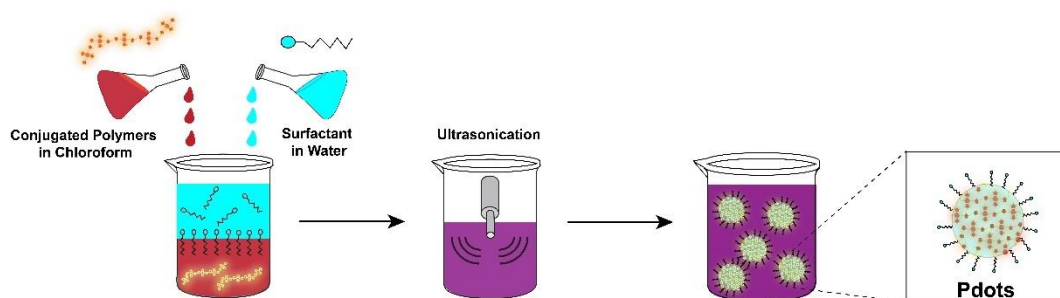


Figure 2.15. Schematic illustration for the preparation of NIR emissive Pdots.

2.5. Characterization Methods

All synthesized intermediate products, monomers, and polymers were characterized by Nuclear Magnetic Resonance (NMR) spectroscopy (Figure 2.16).



Figure 2.16. Varian NMR instrument (400 MHz).

The absorbance and fluorescence properties of polymer products (P1-10) and Pdot solutions were characterized using the Horiba Duetta absorbance and emission spectrometer as seen in Figure 2.17.



Figure 2.17. Horiba Duetta absorbance and emission spectrometer.

Pdot solutions were characterized with the Dynamic Light Scattering (DLS) instrument (Figure 2.18), and the hydrodynamic radius and correlation coefficient of the Pdots were determined.



Figure 2.18. Zetasizer Nano ZS, Malvern Instruments.

Atomic force microscopy (AFM) was used to characterize the morphology of Pdots and determine the charge of Pdots with electrostatic force microscopy (EFM) (Figure 2.19). To analyze the NIR emissive Pdots, 10 μL samples were taken from the Pdot solutions and dried by dropping them on the gold surface.

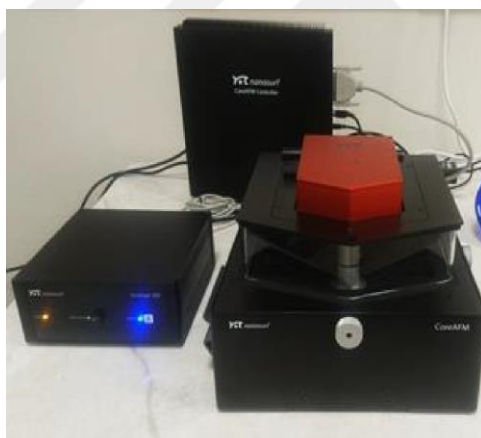


Figure 2.19. Nanosurf-Core AFM instrument.

Imaging applications of Pdots were performed to image cells and 3D tumoroids with fluorescence microscopy (Figure 2.20).

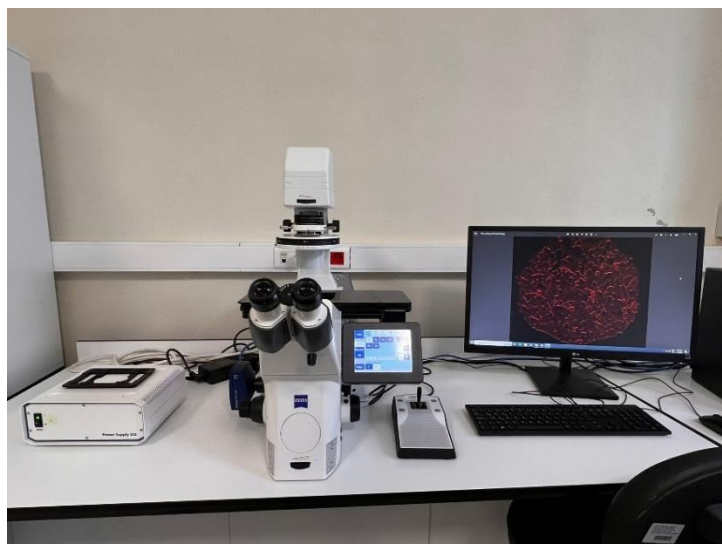


Figure 2.20. Fluorescence microscopy instrument.

2.6. Fluorescence imaging application with Pdots in 2D and 3D cell culture models

Fluorescence microscopy was used to perform cellular-level imaging of NIR emissive Pdots. The objective of this step was to assess the impact of NIR emissive Pdots on various cancer cells by generating 3D tumoroid structures using five different cancer cell lines and visualizing these structures using the Pdots. Given that Pdots effectively stain the cell nucleus, DAPI was used as a marker in both 2D and 3D cultures to benefit as a control group. In this part, the studies were detailed as seen below;

- Optimization and characterization of imaging parameters with NIR emissive Pdot in standard 2D cell culture

In this stage, HeLa (human cervical epithelial adenocarcinoma cell), MDA-MB-231 (human breast adenocarcinoma cell, estrogen-independent), MCF-7 (human breast adenocarcinoma cell, estrogen-dependent), SH-SY5Y (human neuroblastoma cell), PC-12 (rat adrenal gland cell) were used in 2D cell culture studies. To increase the number of cells, the cells were planted on the flask and kept in DMEM medium (GIBCO) containing L-glutamine (10% fetal bovine serum and 1% penicillin/streptomycin) for 2-

3 days. During this process, the cells were stored in a standard incubator (37°C, 5% CO₂) and removed with trypsin after proliferating (confluent) to cover the entire area. After trypsin was inactivated in the serum medium, centrifugation was applied to remove the trypsin-containing medium and collect the cells. The obtained cells were planted in 96 microplates to optimize the Pdot concentration. They were incubated for 24 hours. After the cells adhered to the surface, Pdot was added to the microplate and incubated based on the concentrations (40-100µM) in our previous study (Ozenler et al. 2019) and the previously used protocols. After incubation, it was analyzed under a fluorescent microscope to observe its interaction with cells. Viability experiments were performed to investigate the effect of Pdot on cell growth. Additionally, Pdot localization was examined for five cell lines by labeling the cell nucleus and cytoplasm using DAPI and Calcein Green after Pdot incubation.

- Optimization and characterization of imaging parameters with Pdot in 3D tumoroid models

HeLa, MDA-MB-231, MCF-7, SH-SY5Y, and PC-12 cells were used in 3D tumoroid models to obtain spheroid structure by the hanging drop method.⁷⁹ The obtained 3D tumoroid spheroids were visualized with NIR emissive Pdot. In spheroid formation, the number of cells was first optimized, and the optimum number of cells was determined, where no diffusion limit was observed within the range of cell numbers used. 3D cell culture experiments were continued using the Pdot concentration optimized in 2D cell culture. First, the number of cells was optimized. Then, the minimum interaction time of Pdot with spheroids was looked at for five different cancer cell lines. Pdot penetration behavior was examined in five different cancer cell lines by labeling the cell nucleus and cytoplasm using DAPI and Calcein Green.

- Experiment Design

1. Optimization of Pdot concentration in 2D cell culture

NIR emissive Pdots was applied to cancer cells seeded in 96 microplates in the concentration range (40–100 µM), and its interaction with the cells was examined under fluorescence microscopy after 24 hours of incubation. Experiments were continued with

the lowest concentration that was taken into the cell, and thus fluorescence labeling was observed.

2. Investigation of the effect of Pdot on different cell numbers in 2D cell culture

Five different cell lines are inoculated separately into 96 microplates with a cell count range of 1×10^4 – 5×10^4 and incubated for 24 hours, and it was investigated whether cell density has an effect on fluorescence imaging with Pdot.

3. Determination of minimum interaction time for fluorescence imaging with Pdot in 2D cell culture

At this stage, it was investigated whether there is a difference in the minimum interaction time for fluorescence imaging of different cancer cells with the Pdot. To determine how long it takes for Pdot to be absorbed or interact into the cell and the difference in fluorescence intensity depending on time, cancer cells in the optimized number of cells are seeded into 96 microplates and analyzed by imaging with a fluorescence microscope in 60-minute periods.

4. Characterization of Pdot-applied 2D cells

The region where Pdot was located within the cell (nucleus, cytoplasm, organelle, etc.) was determined by fluorescence staining of organelles in parallel for five different cancer cell lines. Additionally, cell growth and cell viability analyses were performed on Pdot-applied cell lines to determine the toxicity of the applied Pdot. Cell growth experiments were performed with the Alamar Blue method. The cell cytoplasm was marked with Calcein Green, the cell nucleus was labeled with DAPI, and fluorescence microscopy analysis was performed in parallel with Pdot. Thus, the region where Pdot was located in the cell and its penetration behavior were investigated.

5. Optimization of the number of cells in a 3D cell culture (3D tumoroid) model

3D tumoroid spheroids were obtained by the hanging drop method. In the hanging drop method, which is a practical method, the desired cell concentration is first collected with drops of 10–30 μL volume and left on the inside of the petri dish lid, leaving a certain distance between them. Then, the drop is placed on the petri dish

containing PBS. A PBS hydration circle is created in the Petri dish. At this stage, the droplets are held on the lid by surface tension. The cells in the drop come together under gravity and form a 3D spheroid structure as a result of cell-cell interactions.^{79,80} The literature reports that the formation time of spheroids varies depending on the cell line, and the formation time of spheroids was determined individually for each of the five cell lines used in this step. The size of the spheroid structure obtained varies in direct proportion to the number of cells used. When the spheroid size generally exceeds 200 μm , oxygen, nutrients, and growth factors cannot reach the inner region of the spheroid, resulting in a concentration gradient. As a result, a dead cell zone forms in the inner region of the spheroid structure.⁸¹ To prevent this phenomenon, called diffusion limitation, the number of cells must be optimized. For this purpose, trials are made in the range of 1×10^3 – 2.5×10^4 cells to find the appropriate number of cells. Thus, when Pdot is applied in cell viability experiments, it can be understood whether there is a cytotoxic effect specifically caused by Pdot.

6. Determination of minimum interaction time for fluorescence imaging with Pdot in a 3D tumoroid model

At this stage, it was investigated whether there is a difference in the minimum interaction time for imaging with Pdot of 3D spheroid structures created from different cancer cells. To find out how long it takes for Pdot to enter or interact with the cells and how the fluorescence intensity changes over time, the hanging drop method was used to obtain spheroids with the optimized number of cells. These spheroids were then moved to 96 microplates and the optimized concentration of Pdot was added to each well. Afterwards, analysis was carried out by imaging with a fluorescence microscope for 60-minute periods. Additionally, the results obtained were compared with the results of time optimization for 2D cell culture.

7. Characterization of Pdot applied 3D tumoroid spheroids

After the spheroids were incubated in the medium containing NIR emissive Pdots, the cell cytoplasm (green) and cell nucleus (blue) were marked along with Pdot by Calcein Green and DAPI staining, respectively. MitoView™ was used to visualize the mitochondria. Green The spheroids are then fixed using 4% paraformaldehyde.

Subsequently, Pdot localization and penetration are investigated by imaging the nucleus, Pdot, and cell cytoplasm with fluorescence microscopy analysis.

2.7. Karhunen-Loève Transform (KLT) Analysis

KLT, an abbreviation for Karhunen-Loève Transform, is a mathematical method used for signal processing and feature extraction, specifically in the field of image compression and reconstruction. To examine spheroids, KLT (Karhunen-Loève Transform) was used to extract particular features or properties of the Mitoview and Pdots distributions that are essential for investigation. Through the application of KLT analysis, we were able to utilize fluorescence microscopy images more effectively, while nevertheless maintaining crucial spatial information. The KLT analysis was carried out using the FIJI software (IMAGE J2, version 2.14.0/1.54f). In summary, microscope images in PNG format were exported to the FIJI software and subsequently examined using Color Inspector 3D (v2.5). The original set values were maintained for the C0, C1, and C2 axes, as well as for brightness, contrast, saturation, and color rotation. The analysis of spheroid photos involved examining a set region of 14329 pixels, as depicted in Figure 2.21. This was done using the FIJI Color Inspector 3D (v2.5) tool. To summarize, our objective is to determine the primary patterns or sources of variation in the distribution of Mitoview and Pdots within spheroids. Therefore, it is worthwhile to investigate the use of KLT to demonstrate colocalization in different types of spheroid models.

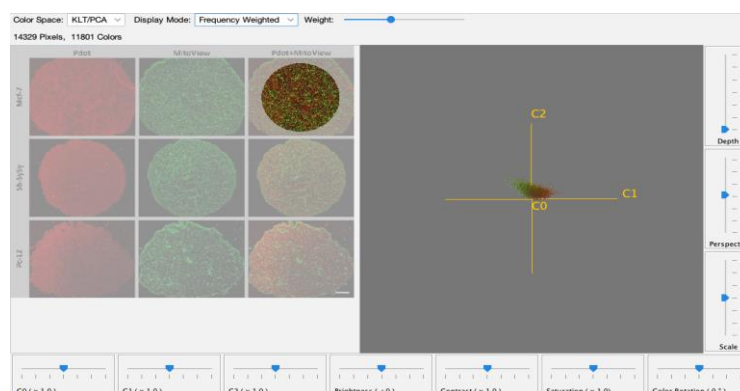


Figure 2.21. Screen shot of the FIJI Color Inspector 3D (v2.5) and KLT analysis cell spheroids.

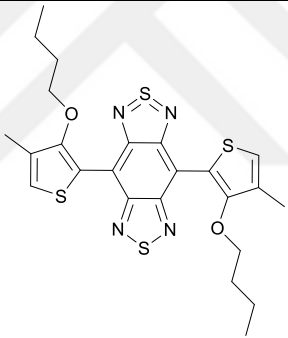
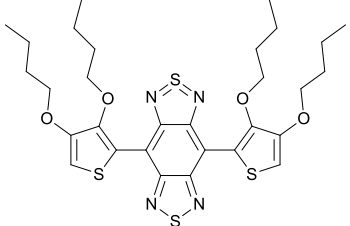
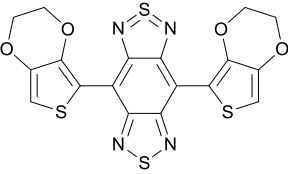
CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Characterization of donor-acceptor-donor based monomers

The monomers shown in Table 3.1 were synthesized and characterized. Characterizations were completed with ^1H , ^{13}C NMR, and mass spectroscopy, and the proposed structures were confirmed.

Table 3.1. Synthesized Monomers

Short names of synthesized monomers	Molecular weight (g/mol)	Yield (%)	Chemical structure	Explanation
M1	530,74	51.4		Monomer 1 containing BuMT-BBT
M2	660.93	55		Monomer 2 containing DiBuT-BBT
M3	474.54	60		Monomer 3 containing EDOT-BBT

3.1.1. Characterization of M1

BuMT was obtained as 0.60 g of light-yellow oil with a yield of 63%. In the synthesis study conducted by Iwatsuki et al. (1993), a synthesis close to our protocol was performed, and the yield was calculated at 48%. In another reference article, the yield was calculated at 52% as a result of a synthesis study similar to our protocol by Tanaka et al.⁸² The BuMT precursor was synthesized with higher efficiency than the referenced articles. ¹H NMR analysis was performed to confirm the structure (Figure 3.1). The spectrum confirms BuMT's structure. ¹H NMR (400 MHz, CDCl₃) δ 6.81 – 6.79 (m, 1H), 6.11 (d, J = 3.2 Hz, 1H), 3.92 (t, J = 6.4 Hz, 2H), 2.08 (s, 3H), 1.79 – 1.72 (m, 2H), 1.52 – 1.43 (m, 2H), 0.96 (t, J = 7.4 Hz, 3H). Also, ¹³C NMR analysis of BuMT was given in Figure 3.2. The signals obtained in NMR support the structure. ¹³C NMR (101 MHz, CDCl₃) δ 156.3, 129.3, 119.7, 95.8, 69.7, 31.3, 19.3, 13.9, 12.7.

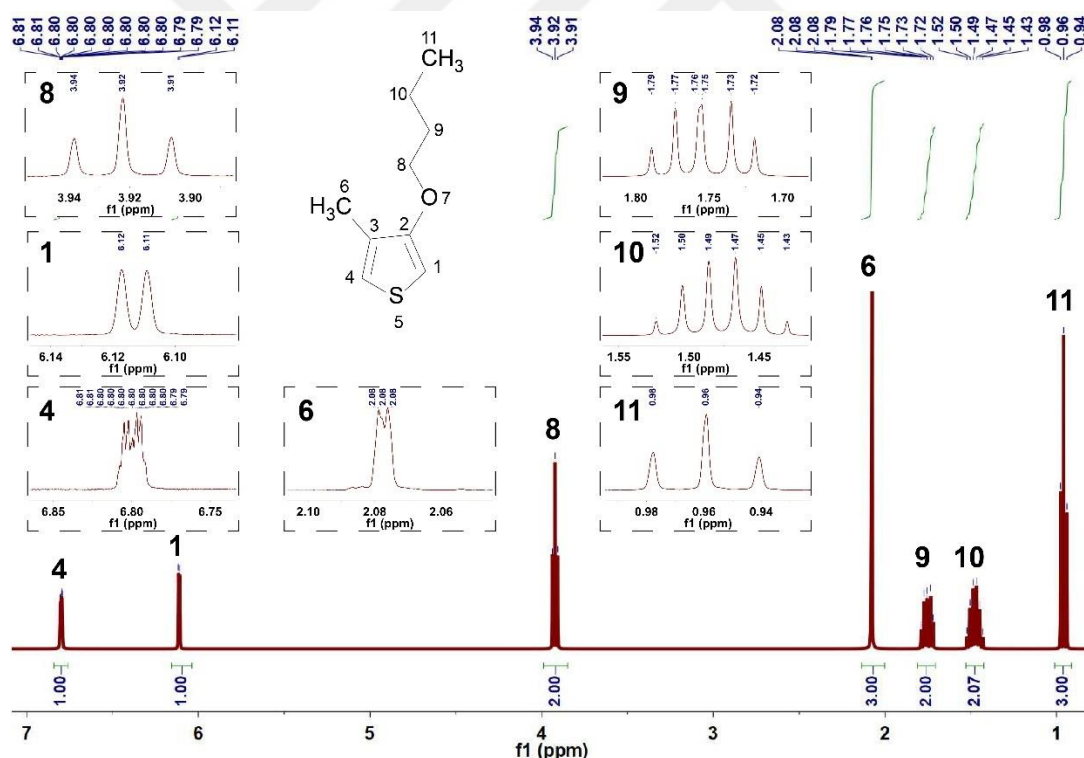


Figure 3.1. ¹H NMR spectrum of BuMT.

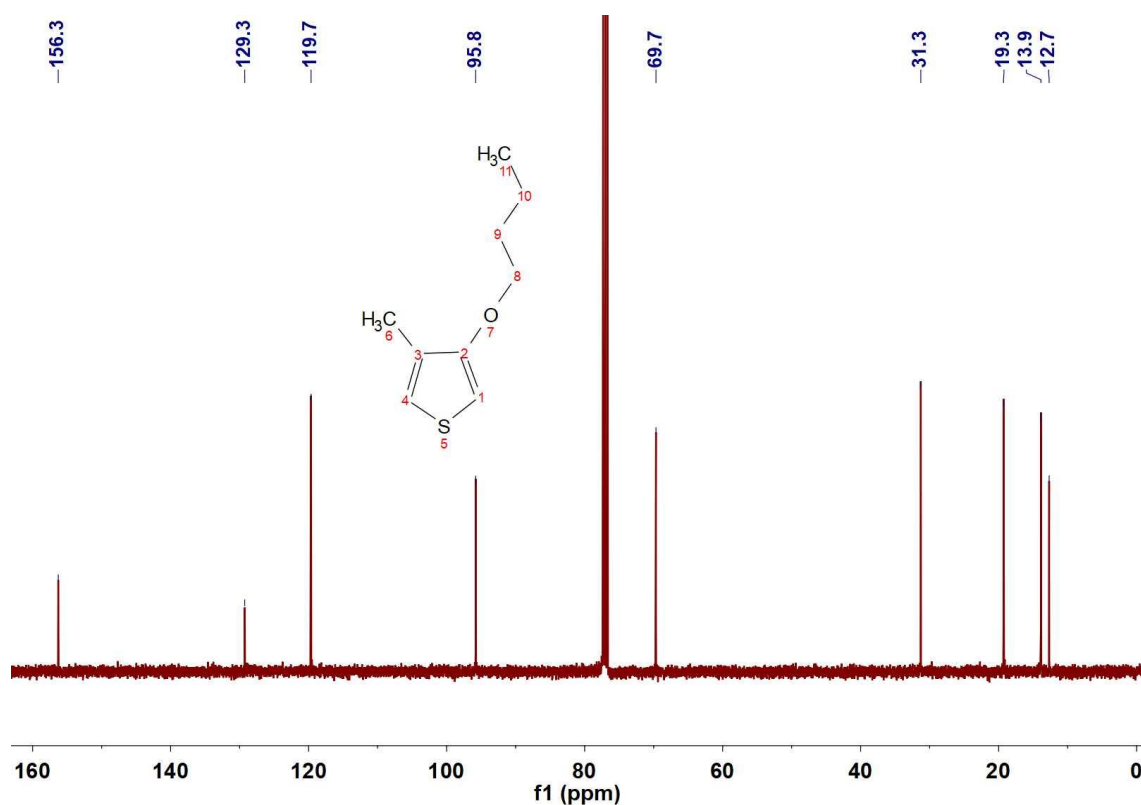


Figure 3.2. ^{13}C NMR spectrum of BuMT.

M1 (Bis-3-butoxy-4-methylthiophene benzobisthiadizole), which is dark blue, was obtained in 51.4% yield as 90 mg. The monomer was characterized by ^1H NMR, as shown in the Figure 3.3. ^1H NMR (400 MHz, CDCl_3) δ 6.55 (s, 2H), 4.06 (t, J = 6.4 Hz, 4H), 2.03 (s, 6H), 1.86 – 1.79 (m, 4H), 1.58 – 1.49 (m, 4H), 1.00 (t, J = 7.4 Hz, 6H).. In addition, ^{13}C NMR analysis was performed for M1 (Figure 3.4). ^{13}C NMR (101 MHz, CDCl_3) δ 156.4, 155.7, 153.0, 152.8, 132.3, 131.0, 128.3, 123.5, 117.1, 116.0, 115.3, 99.3, 73.3, 69.6, 31.8, 31.3, 27.8, 26.8, 19.4, 18.7, 17.5, 13.9, 13.6, 13.2. The signals obtained in NMR analyses confirm M1's structure.



44

These results indicate a purity of over 90% for the targeted M1. These results indicated that M1 was polymerizable, leading to the continuation of the oxidative polymerization steps.

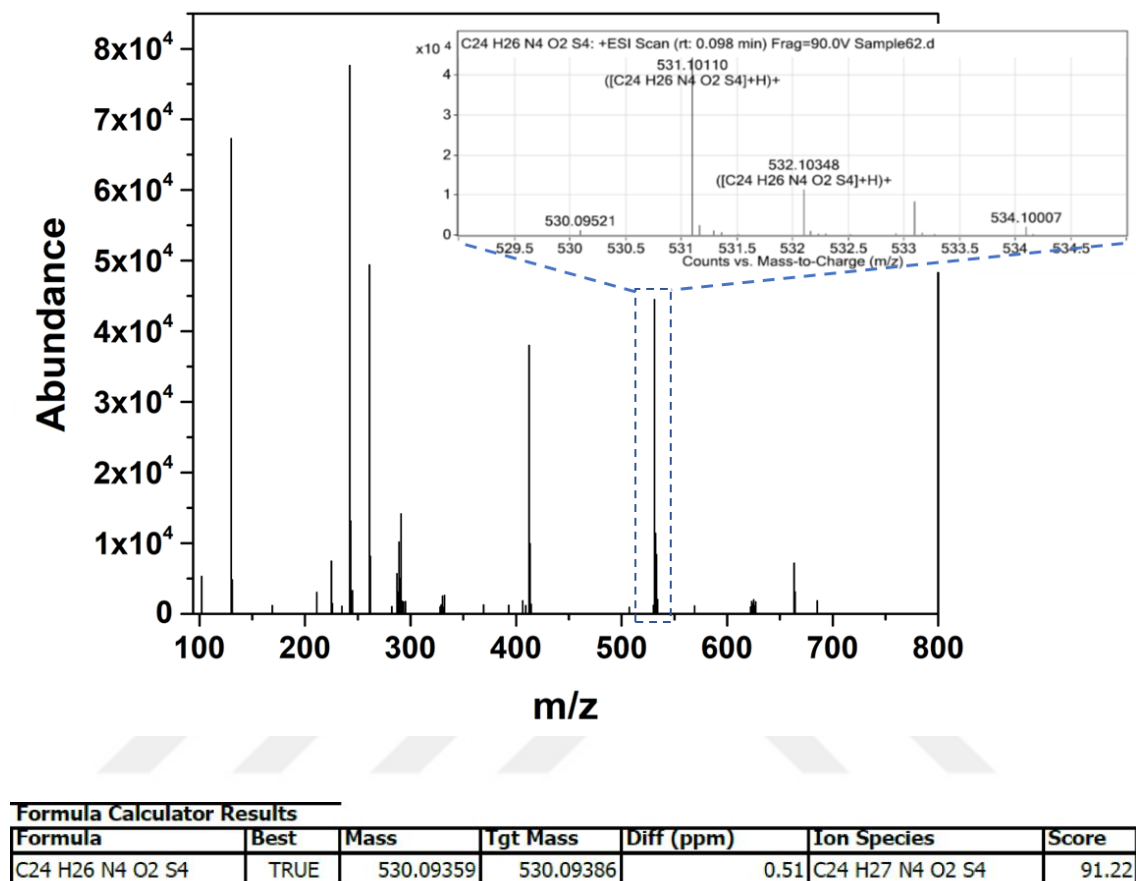


Figure 3.5. MS spectrum of M1.

Optical characterization of the M1 was carried out. Figure 3.6 shows the absorption and emission spectra of M1 with λ_{max} values of 616 nm and 810 nm, respectively. The results show that M1 has an emission in the NIR region and a high stoke shift of 194 nm.

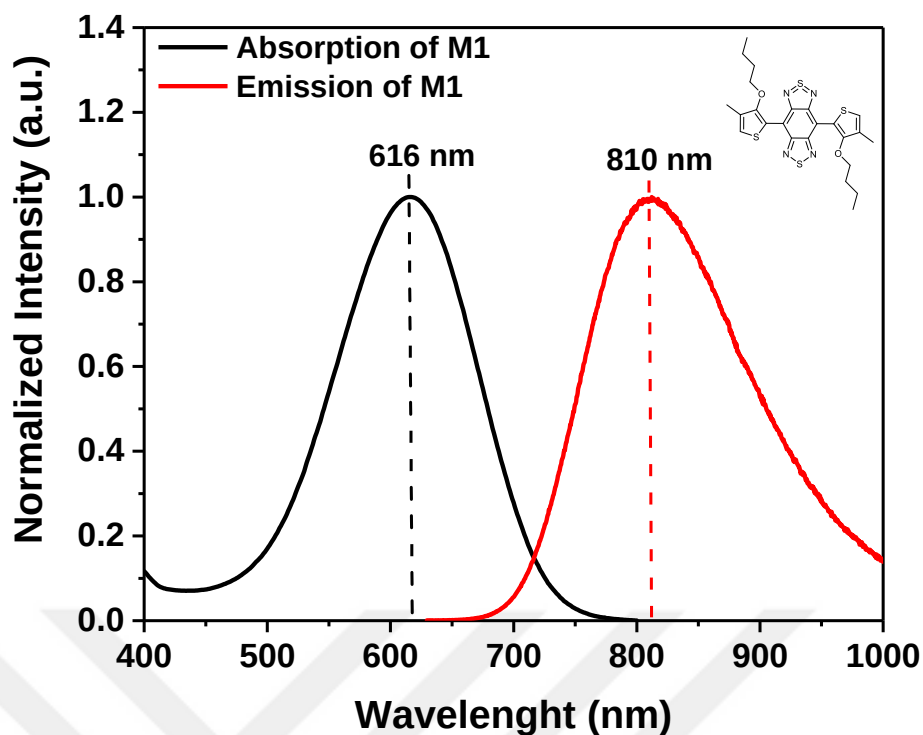


Figure 3.6. Absorption and emission spectra of D-A-D M1 in chloroform.

3.1.2. Characterization of M2

Firstly, DiBuT was synthesized as a donor source of D-A-D based M2. The DiBuT was obtained as 0.50 g of light-yellow oil with a yield of 63%. Then, the compound was analyzed via ^1H NMR (Figure 3.7). ^1H NMR (400 MHz, CDCl_3) δ 6.16 (s, 2H), 3.98 (t, J = 6.7 Hz, 4H), 1.80 (ddt, J = 8.9, 7.9, 6.6 Hz, 4H), 1.53 – 1.43 (m, 4H), 0.97 (t, J = 7.4 Hz, 6H). Also, ^{13}C NMR analysis performed for the DiBuT as seen in Figure 3.8. ^{13}C NMR (101 MHz, CDCl_3) δ 147.6, 96.8, 70.2, 31.1, 19.2, 13.8.

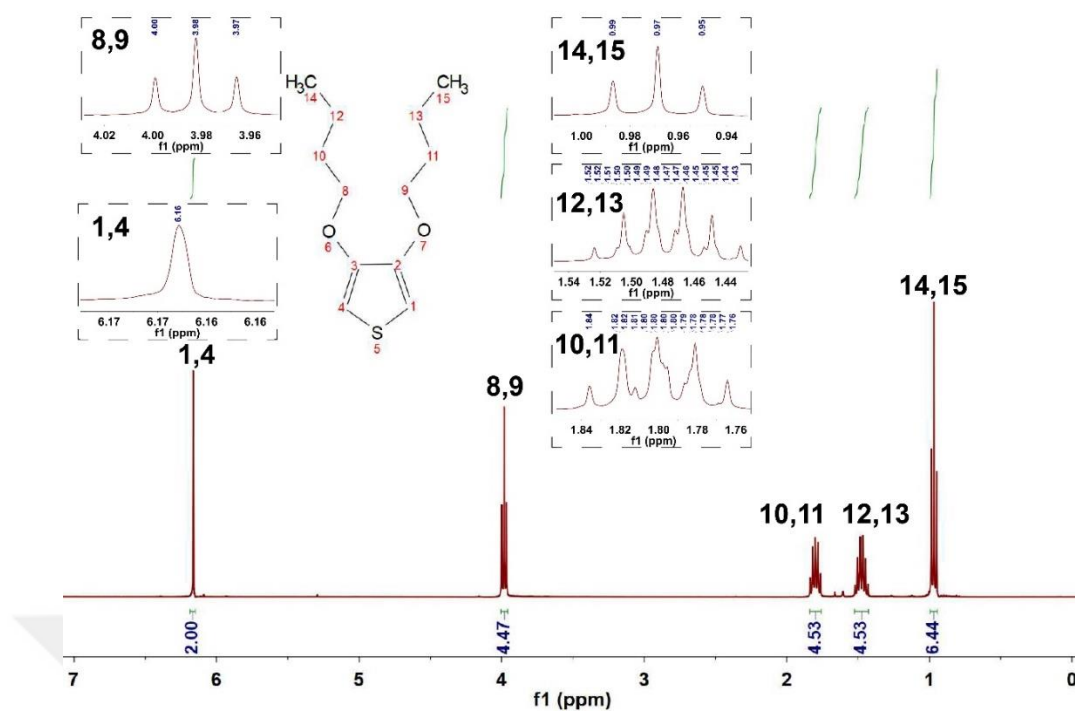


Figure 3.7. ^1H NMR spectrum of DiBuT.

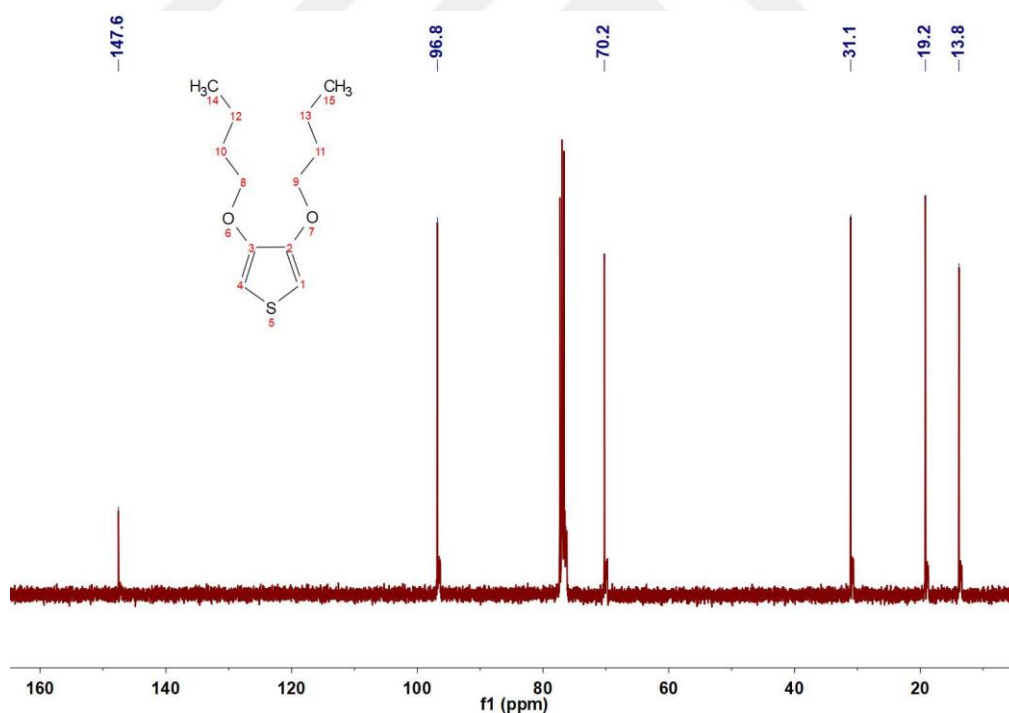


Figure 3.8. ^{13}C NMR spectrum of DiBuT.

After DiBuT was characterized, one-sided stannylated DiBuT was synthesized to be used in the Stille coupling reaction for obtaining D-A-D-based M2. Accordingly,

the synthesized precursor was analyzed via ^1H NMR. The spectrum confirms the structure of one-sided stannylated DiBuT with characteristic peaks (Figure 3.9). ^1H NMR (400 MHz, CDCl_3) δ 6.40 (s, 1H), 3.96 – 3.91(dt, J = 9.1, 6.6 Hz, 4H), 1.81 – 1.64 (m, 4H), 1.60 – 1.39 (m, 16H), 1.37 – 1.26 (m, 6H), 1.11 – 1.05 (m, 6H), 0.95 (td, J = 7.4, 1.6 Hz, 9H).

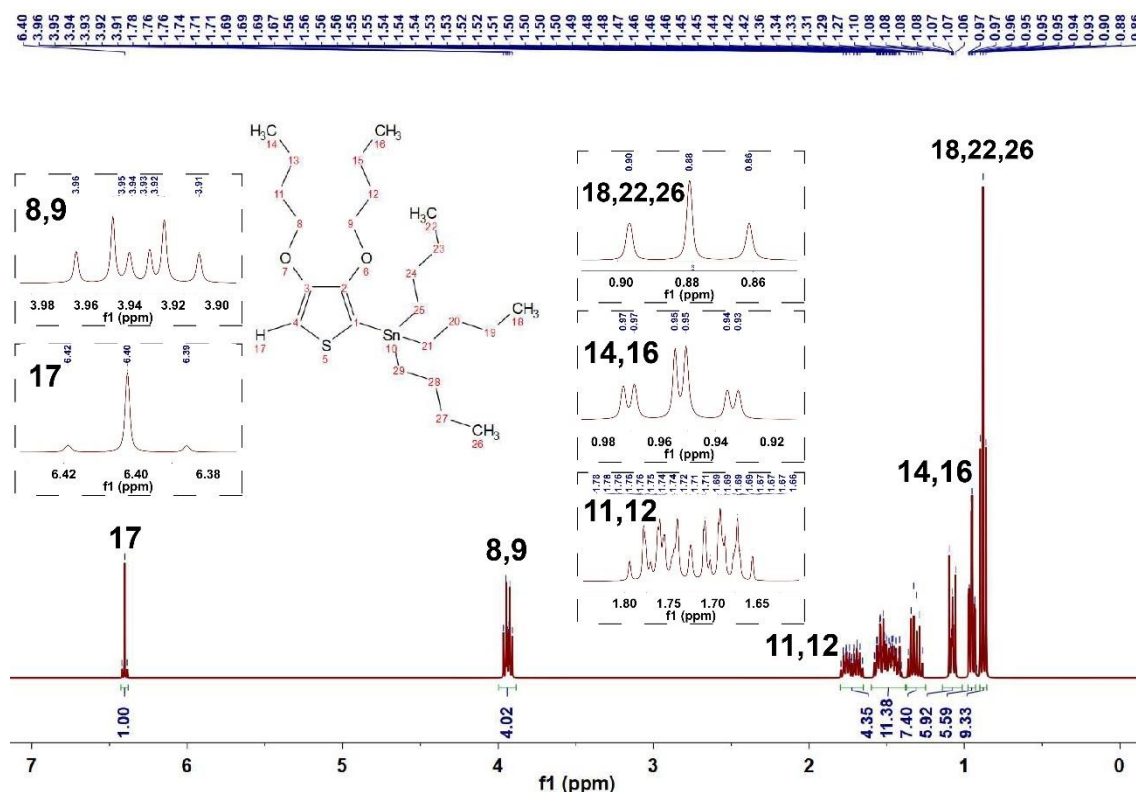


Figure 3.9. ^1H NMR spectrum of one-sided stannylated DiBuT.

The one-sided stannylated DiBuT was coupled with an acceptor unit BBT to obtain D-A-D based M2. The monomer was characterized by ^1H NMR. According to the ^1H NMR spectra, the structure of the M2 was confirmed by the characteristic signals. ^1H NMR (400 MHz, CDCl_3) δ 6.55 (s, 2H), 4.08 (dt, J = 9.2, 6.5 Hz, 8H), 1.88 – 1.79 (m, 4H), 1.59 – 1.41 (m, 8H), 1.39 – 1.31 (m, 4H), 1.00 (t, J = 7.4 Hz, 6H), 0.62 (t, J = 7.4 Hz, 6H). Also, ^{13}C NMR analysis was performed for the M2 (Figure 3.11). ^{13}C NMR (101 MHz, CDCl_3) δ 153.0, 150.3, 146.2, 116.9, 114.7, 99.6, 72.2, 69.8, 31.9, 31.3, 29.7, 19.3, 18.7, 13.8, 13.5.

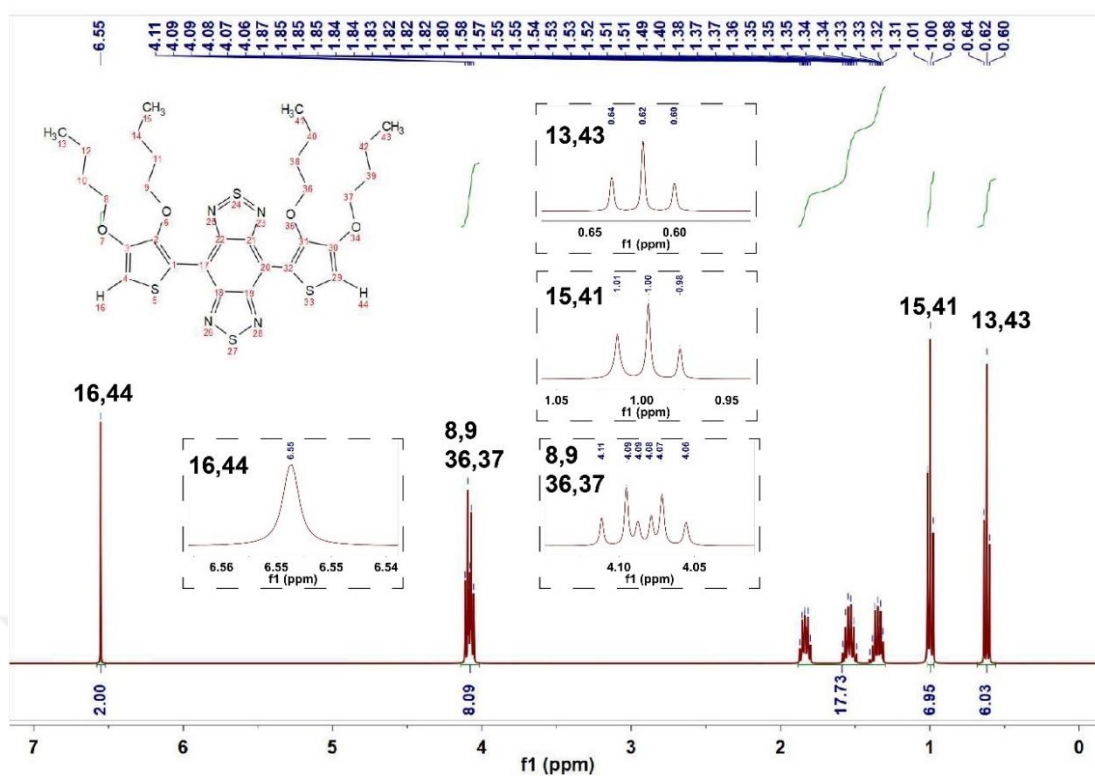


Figure 3.10. ^1H NMR spectrum of Monomer 2.

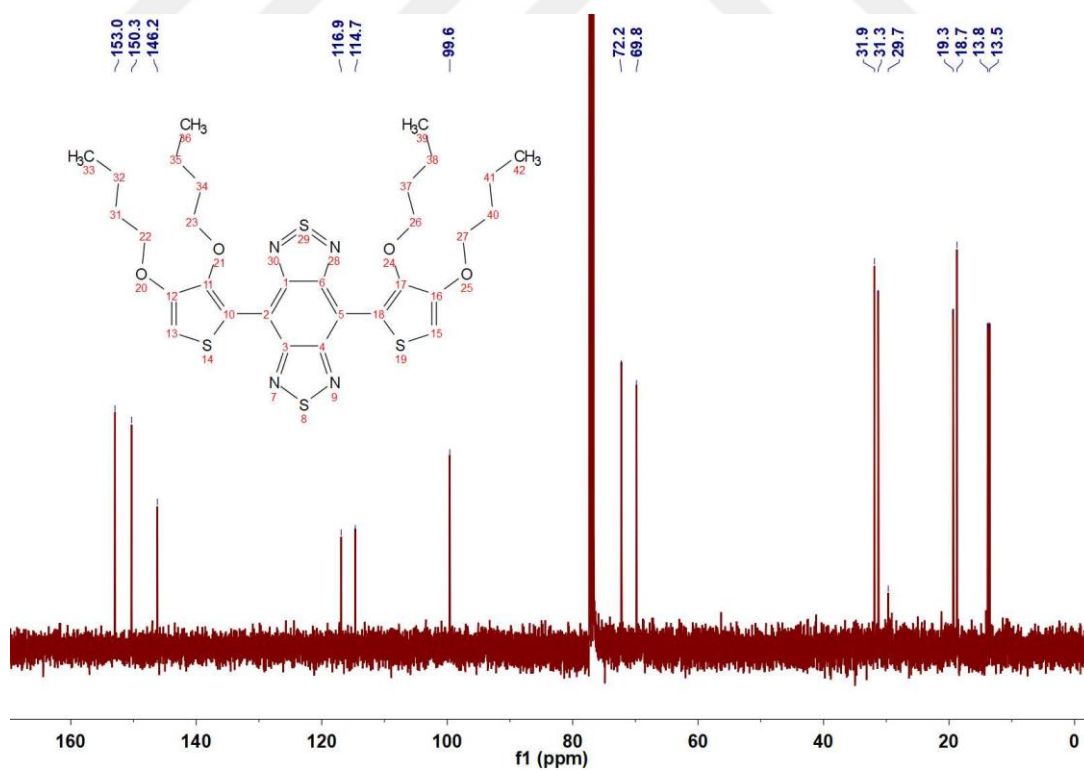


Figure 3.11. ^{13}C NMR spectrum of the M2.

Optical characterization of the M2 was carried out. Figure 3.12 shows the absorption and emission spectra of M2 with λ_{max} values of 615 nm and 820 nm, respectively. The results show that M2 has an emission in the NIR region and a high stoke shift of 205 nm.

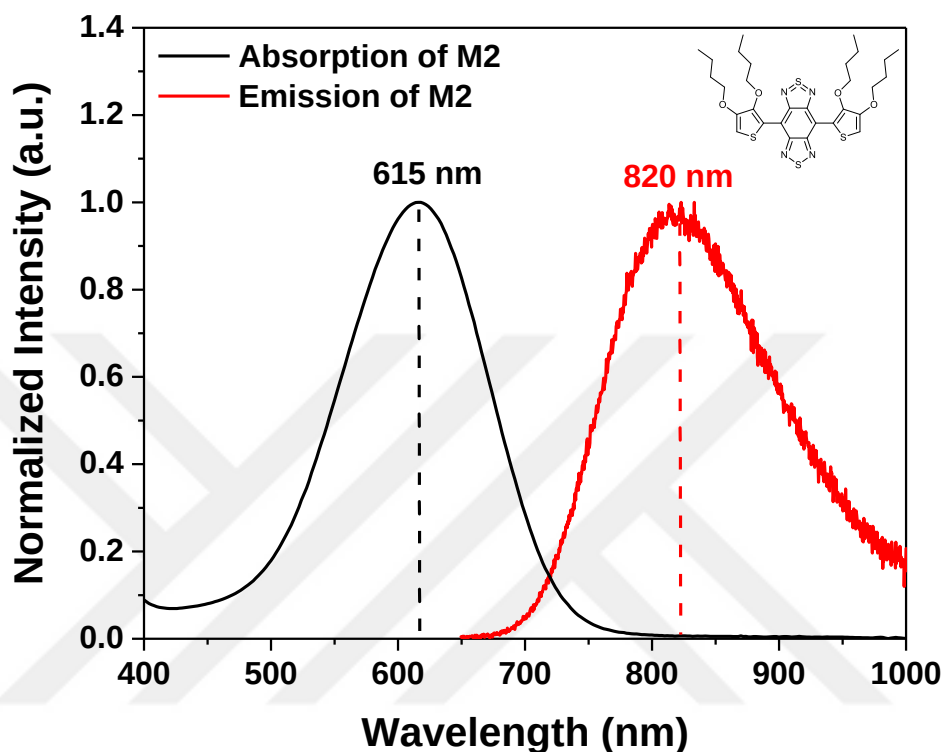


Figure 3.12. Absorption and Emission spectra of M2 in chloroform.

3.1.3. Characterization of M3

EDOT was purchased as a donor source of D-A-D based M3. After EDOT was obtained, one-sided stannylated EDOT was synthesized to be used in the Stille coupling reaction for obtaining D-A-D-based M3. Accordingly, the synthesized precursor was analyzed via ^1H NMR. The spectrum confirms the structure of one-sided stannylated EDOT with characteristic peaks (Figure 3.13). ^1H NMR (400 MHz, CDCl_3) δ 6.58 (d, J = 0.6 Hz, 1H), 4.16 (q, J = 0.9 Hz, 4H), 1.64 – 1.51 (m, 6H), 1.42 – 1.25 (m, 6H), 1.15 – 1.06 (m, 6H), 0.91 (t, J = 7.3 Hz, 9H).

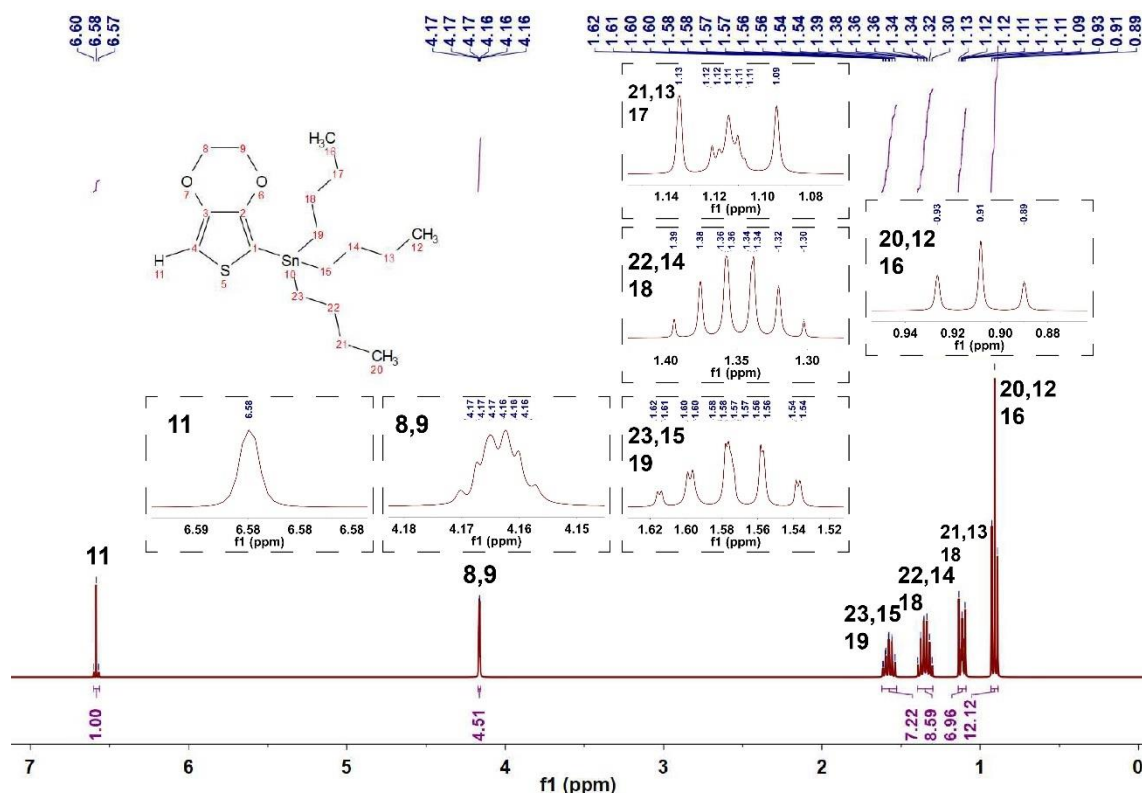


Figure 3.13. ^1H NMR spectrum of one-sided stannylated EDOT.

The one-sided stannylated EDOT was coupled with an acceptor unit BBT to obtain D-A-D based M3. The monomer was characterized by ^1H NMR (Figure 3.14). According to the ^1H NMR spectra, the structure of the M3 was confirmed by the characteristic signals. ^1H NMR (400 MHz, CDCl_3) δ 6.76 (s, 2H), 4.36 – 4.32 (m, 4H), 4.29 – 4.25 (m, 4H). Also, ^{13}C NMR analysis was carried out for the M3, but all characteristic peaks of the monomer were not obtained because of insufficient quantity and analysis time.

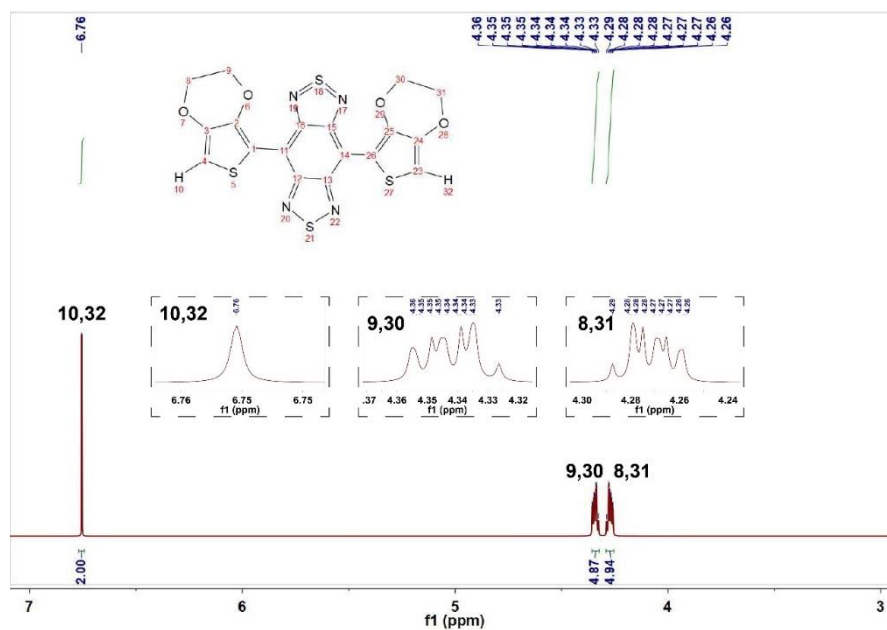


Figure 3.14. ^1H NMR spectrum of Monomer 3.

Optical characterization of the M3 was carried out. Figure 3.15 shows the absorption and emission spectra of M3 with λ_{max} values of 645 nm and 845 nm, respectively. The results show that M3 has an emission in the NIR region and a high stoke shift of 200 nm.

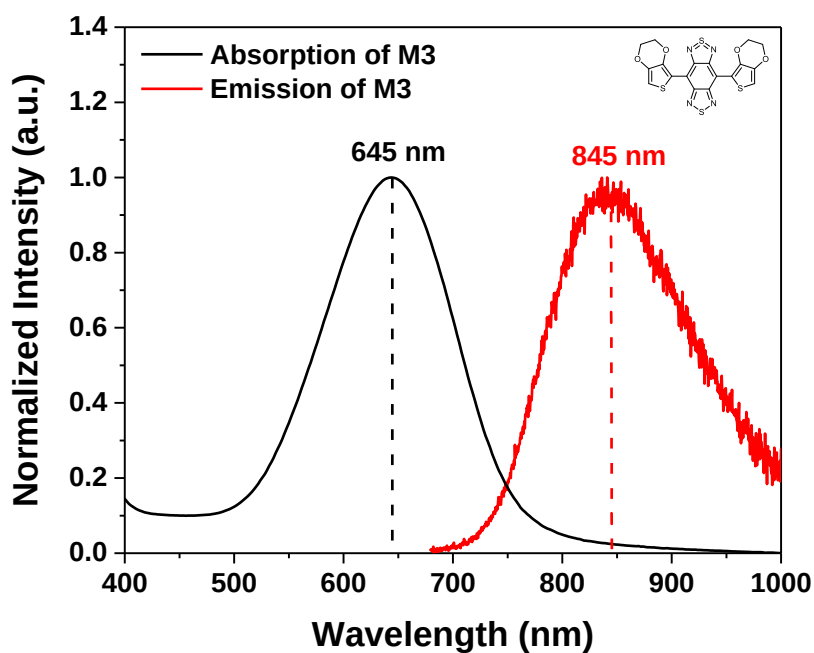
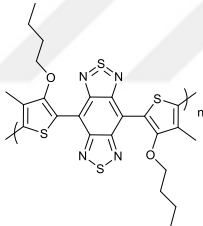
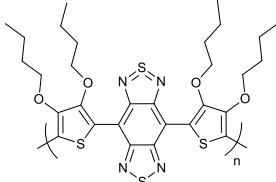
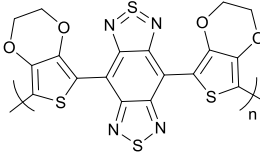


Figure 3.15. Absorption and emission spectra of M3 in chloroform

3.2. Characterization of NIR emissive conjugated polymers

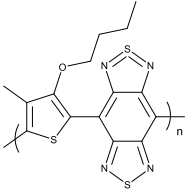
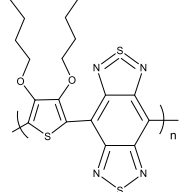
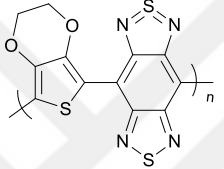
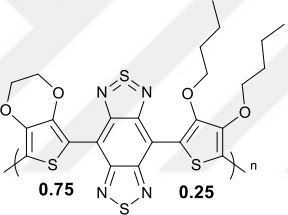
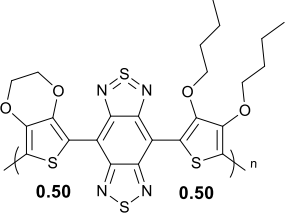
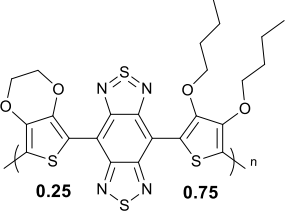
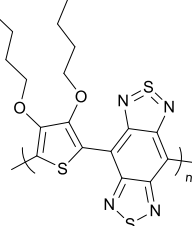
NIR emissive conjugated polymers were synthesized using three different polymerization protocols: oxidative, direct arylation, and stille polymerization. These polymers were named P1–10, and they were shown in Table 3.2 with name, chemical structure, polymerization type, and optic properties. The first three polymers (P1-3) were synthesized via oxidation polymerization. P4 and P5 polymers were synthesized using the DArP protocol. The P6–10 polymers were synthesized via Stille polymerization. In Stille polymerization products, P7-9 polymers were synthesized with different mol ratios of donor sources as an EDOT and DiBuT. The mol ratios of the donor sources were also given in a table. The NIR emissive conjugated polymers were examined in subtitles related to their polymerization types of polymers.

Table 3.2. Synthesized NIR emissive conjugated polymers.

Polymers	Chemical structure of polymers	Polymerization type	Abs and Ems maxima	Explanation
P1		Oxidative Polymerization	λ_{max} , abs: 660 nm λ_{max} , ems: 860 nm	Synthesized 
P2		Oxidative Polymerization	λ_{max} , abs: 630 nm λ_{max} , ems: 856 nm	Synthesized 
P3		Oxidative Polymerization	λ_{max} , abs: 940 nm λ_{max} , ems: -	Synthesized 

(Cont. on the next page)

Table 3.2 (cont.)

Polymers	Chemical structure of polymers	Polymerization type	Abs and Ems maxima	Explanation
P4		Direct arylation Polymerization	λ_{max} , abs: - λ_{max} , ems: -	Could not be synthesized 
P5		Direct arylation Polymerization	λ_{max} , abs: - λ_{max} , ems: -	Could not be synthesized 
P6		Stille Polymerization	λ_{max} , abs: 755 nm λ_{max} , ems: 881 nm	Synthesized 
P7 EDOT-DiBuT 0.75-0.25		Stille Polymerization	λ_{max} , abs: 741 nm λ_{max} , ems: 883 nm	Synthesized 
P8 EDOT-DiBuT 0.50-0.50		Stille Polymerization	λ_{max} , abs: 705 nm λ_{max} , ems: 875 nm	Synthesized 
P9 EDOT-DiBuT 0.25-0.75		Stille Polymerization	λ_{max} , abs: 680 nm λ_{max} , ems: 810 nm	Synthesized 
P10		Stille Polymerization	λ_{max} , abs: 630 nm λ_{max} , ems: 775 nm	Synthesized 

3.2.1. Oxidative Polymerization Products

NIR emissive conjugated polymers were synthesized via oxidative polymerization. Accordingly, three different donor-acceptor-donor based conjugated monomers were prepared as M1, M2, and M3. They were used to obtain their NIR emissive conjugated polymers as P1, P2, and P3. The M1 yielded the P1 (poly(4,8-di-3-butoxy-4-methylthiophenebenzobisthiadiazole)) via oxidation polymerization, which exhibited NIR emission. The resulting P1 was then analyzed using NMR spectroscopy for characterization. The ^1H NMR spectrum of the resultant P1 is shown with M1 in Figure 3.16. The spectrum demonstrates significant alterations, such as a broadening of peaks in the 1.0–2.0 ppm range and the absence of aromatic hydrogens around 6.5–6.6 ppm. These changes are attributed to the successful polymerization process.

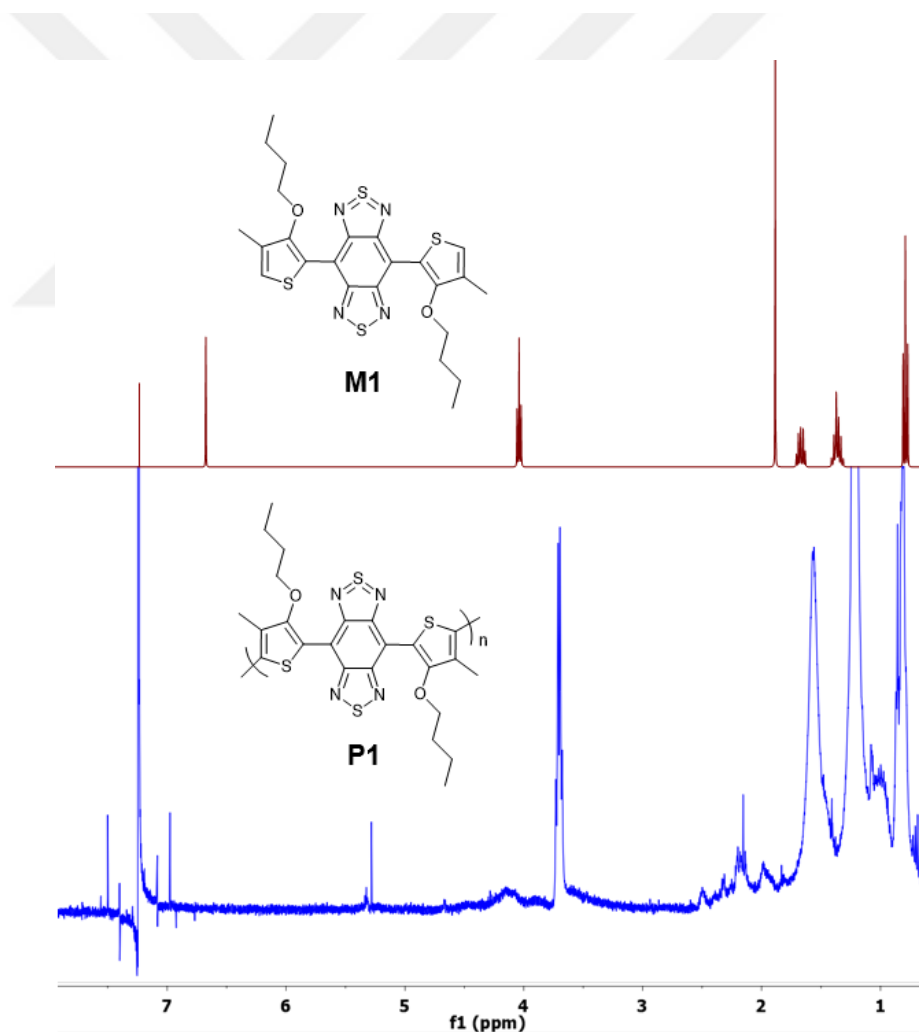


Figure 3.16. Stack ^1H NMR spectrum of NIR emissive P1 with M1.

The optical properties of P1 are illustrated in Figure 3.17, which shows the absorption and emission λ_{max} values of 660 nm and 860 nm, respectively. An appreciable red shift was observed relative to the λ_{max} values of both the monomer (Figure 3.6) and P1, which indicates an elongation of the conjugation length as a consequence of polymerization.

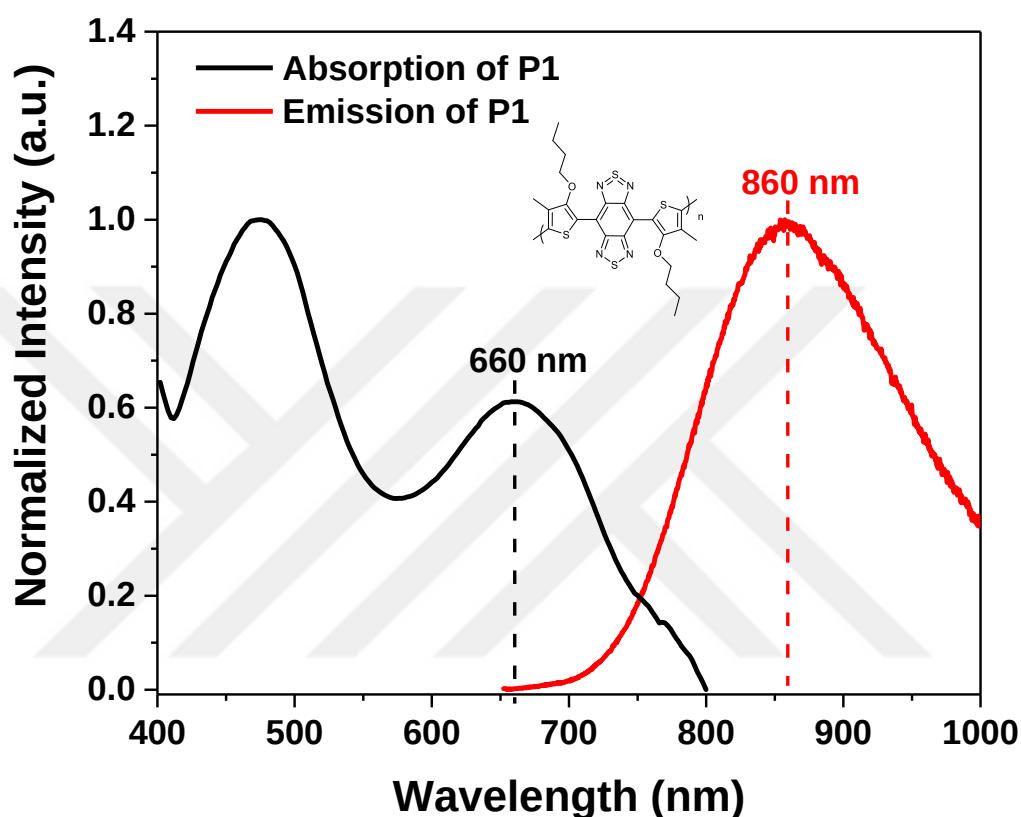


Figure 3.17. Absorption and emission spectra of NIR emissive P1 in chloroform.

Another NIR emissive conjugated polymer is P2 (poly(4,8-3,4-dibutoxythiophenebenzobisthiadiazole)), which was synthesized from M2 via oxidation polymerization. The optical properties of P2 are demonstrated in Figure 3.18, which shows the absorption and emission λ_{max} values of 630 nm and 856 nm, respectively. As compared to P1's optic properties, there is no significant difference. It can be said that the polymerization protocol works properly for the M2.

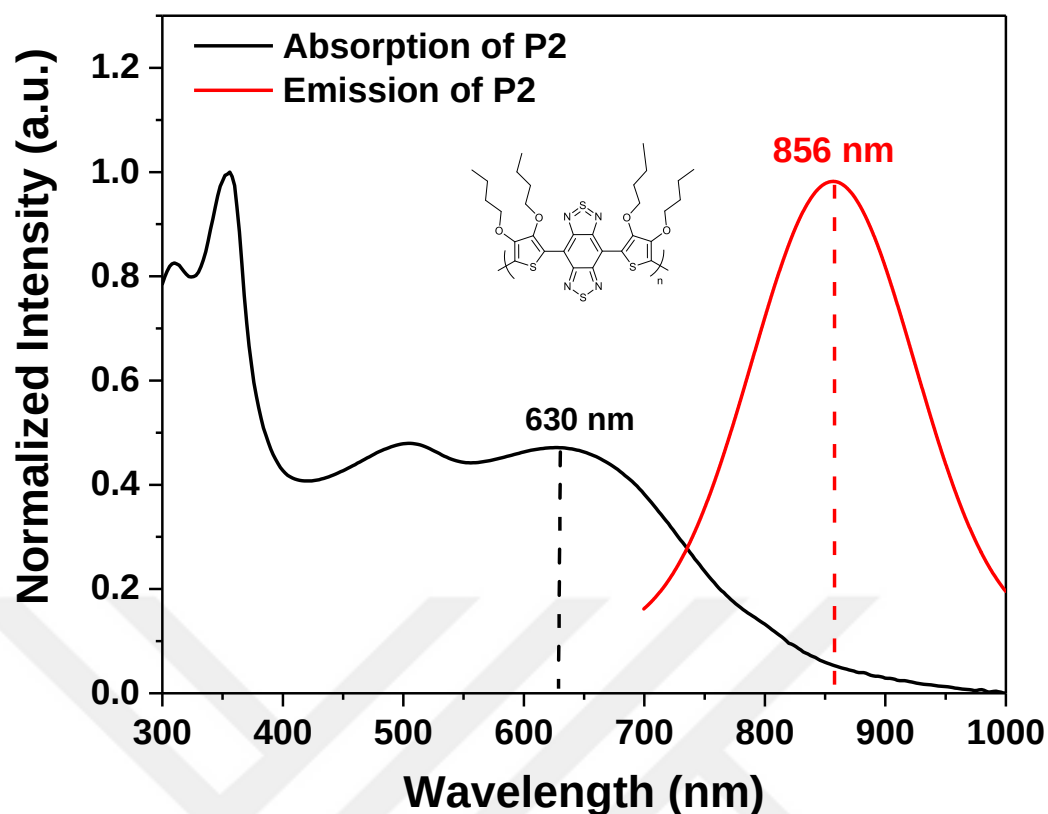


Figure 3.18. Absorption and emission spectrum of P2 in chloroform.

Another NIR emissive conjugated polymer is P3 (poly(4,8-3,4-ethylenedioxythiophenebenzobisthiadiazole)), which was synthesized from M3 via oxidation polymerization. The optical properties of P3 are demonstrated in Figure 3.19, which shows the absorption λ_{max} value of 940 nm. Compared to the other oxidative polymerization products (P1 and P2), the NIR emissive P3 has a significant broad absorption with a high λ_{max} value. I could not characterize the emission optic properties of P3 because we do not have the NIR-II region fluorescence spectroscopy instrument. However, we try to characterize it via our Horiba instrument with an excitation wavelength of 900 nm, as seen in Figure 3.19. The emission maximum value is 957 nm, according to the experiment.

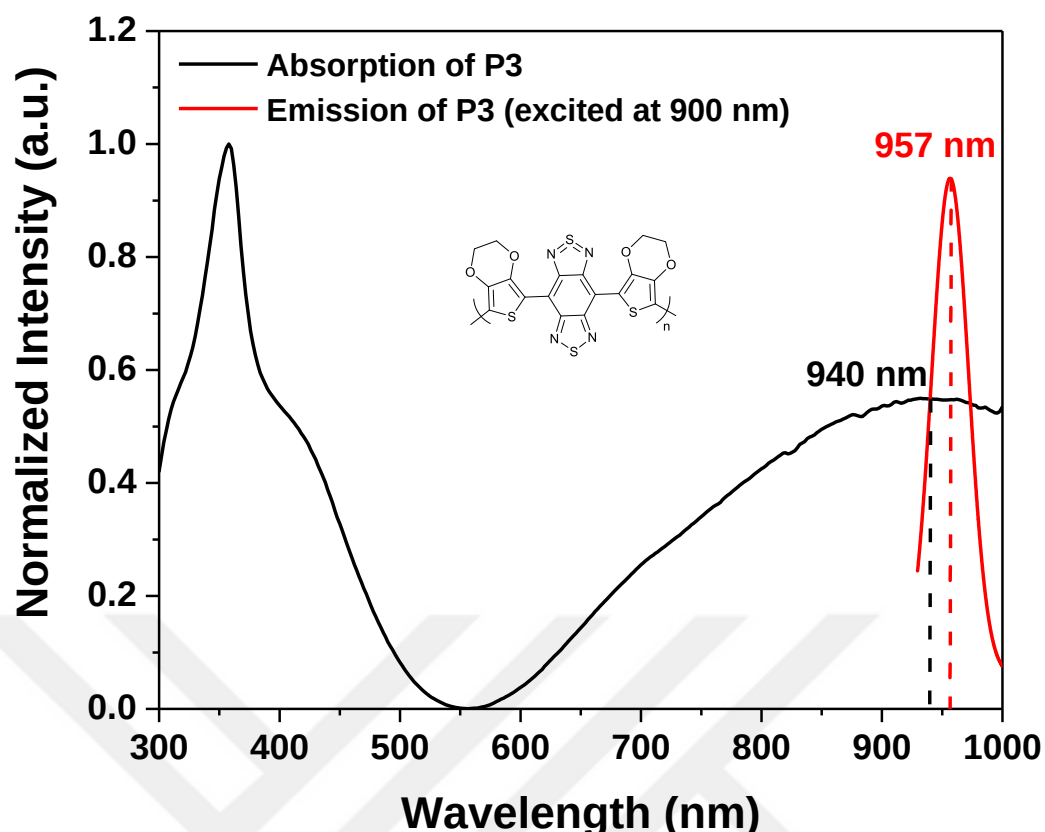


Figure 3.19. Absorption and emission spectrum of P3 in chloroform (excitation wavelength: 900 nm).

As a result of the analysis, P3 has the longest conjugated polymer chain compared to P1 and P2. This is because M3 does not have long aliphatic groups on the thiophene unit, which is why it can easily polymerize. On the other hand, other monomers have some defects in their polymerization capabilities because of their long aliphatic groups (butoxy). To visualize this situation, all oxidative polymerization products's absorption spectra were drawn together to compare their optic properties. As seen in Figure 3.20, their correlation of absorption λ_{max} values is coherent with their polymerization ability of monomer structure. Especially, though the one difference between M1 and M2 is a butoxy group, P1 has a bigger absorption λ_{max} value than P2. Therefore, the results support the above interpretation of the effect of the butoxy group on oxidative polymerization.

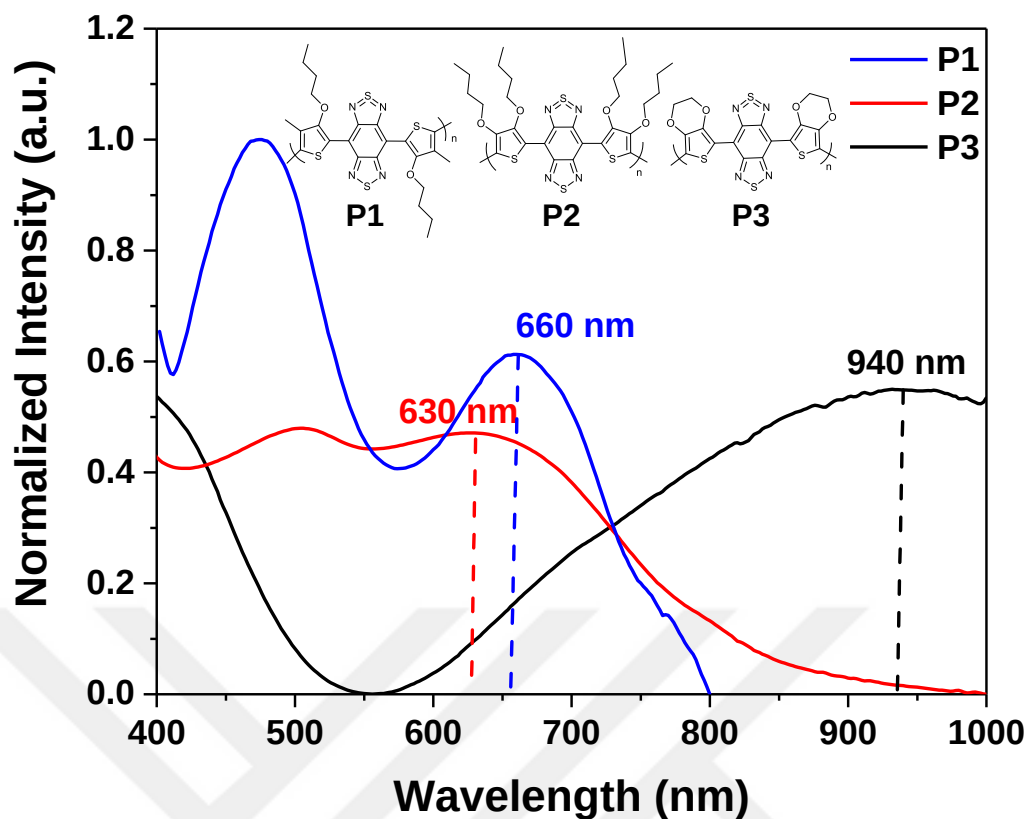


Figure 3.20. Comparison of absorption spectra between oxidative polymerization products (P1, P2, and P3).

3.2.2. Direct Arylation Polymerization Products

NIR emissive conjugated polymers (P4, and P5) were tried to synthesize via direct arylation polymerization. Accordingly, two different donor-acceptor based conjugated polymers were tried to be prepared as P4, and P5. Firstly, DArP protocols were tested using 2-bromo-3-butoxy-4-methylthiophene (Br-BuMT), which was synthesized from BuMT. In this trial, three different DArP protocols were used to synthesize poly BuMT (PBuMT). Then, Br-BUMT was synthesized for use in DArP protocols. The chemical structure of the Br-BuMT was confirmed via ^1H NMR spectroscopy. As seen in Figure 3.21, one-sided bromination was successfully performed on the BUMT. ^1H NMR (400 MHz, CDCl_3) δ 6.80 (dq, $J = 1.7, 0.8$ Hz, 1H), 3.99 (td, $J = 6.5, 0.7$ Hz, 2H), 2.12 (dd, $J = 1.1, 0.6$ Hz, 3H), 1.77 – 1.68 (m, 2H), 1.56 – 1.44 (m, 2H), 0.96 (td, $J = 7.4, 0.6$ Hz, 3H).

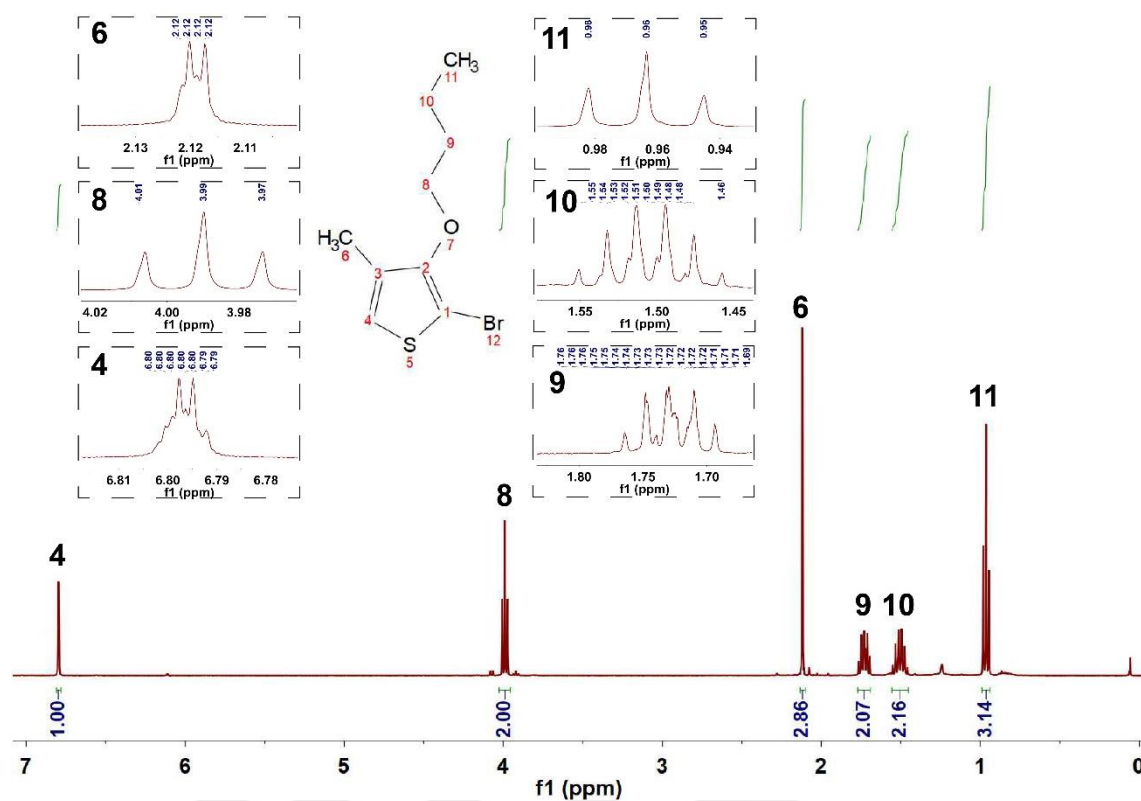


Figure 3.21. ^1H NMR spectrum of the Br-BUMT.

Then, the polymers were obtained using three different DArP protocols. The PBuMT polymers were characterized by ^1H NMR spectroscopy. Figure 3.22 shows the stacked ^1H NMR spectra of the PBuMT polymers, which are nearly identical. The Figure 3.22 supports the polymerization with specific signals, which are the absence of a proton on the thiophene and the broadening of the aliphatic peaks between 1.00 and 2.00 ppm.

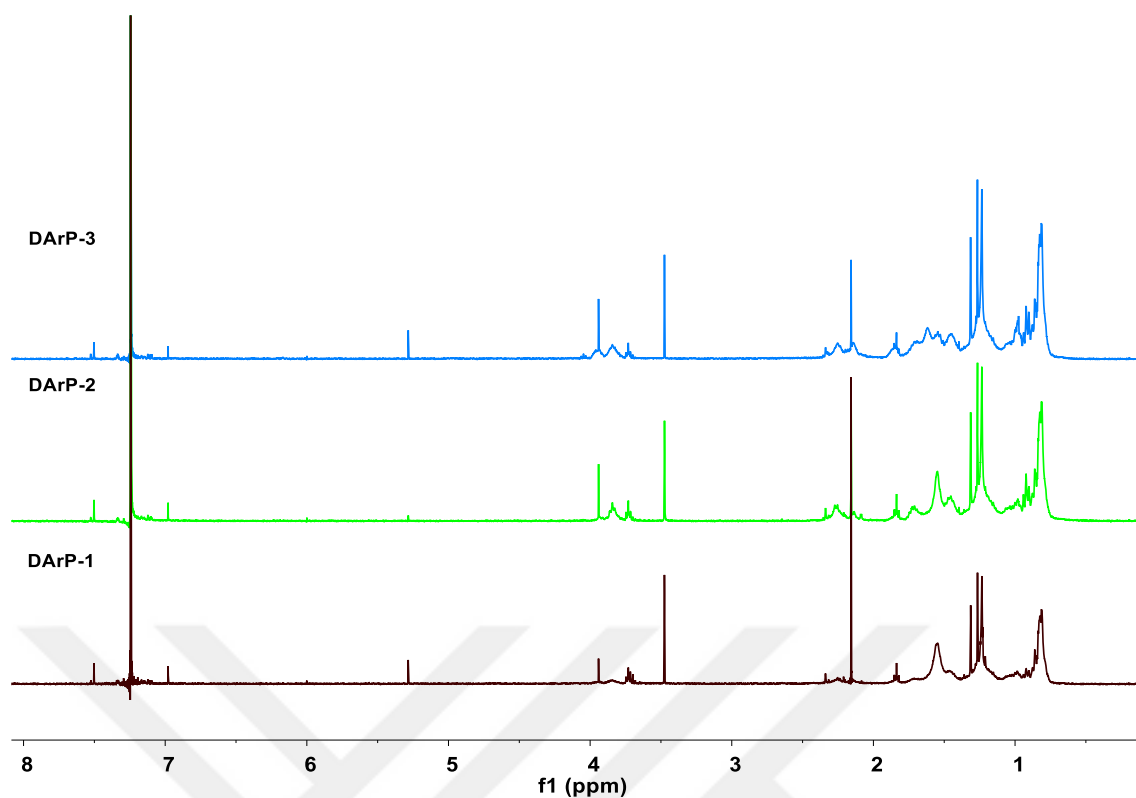


Figure 3.22. Stacked ¹H NMR spectra for PBUtMT via three different DArP protocols.

Afterward, UV-visible spectroscopy was used to characterize the optic properties of PBUtMT polymers. Figure 3.23 demonstrates the absorption spectrum for PBUtMT via the DArP-1, DArP-2, and DArP-3 protocols. As seen in Figure 3.23, PBUtMT has a maximum wavelength of 422 nm for all DARP protocols. The results show that the PBUtMT has nearly the same conjugation length. Therefore, they demonstrated the same spectral behavior in the absorbance analysis.

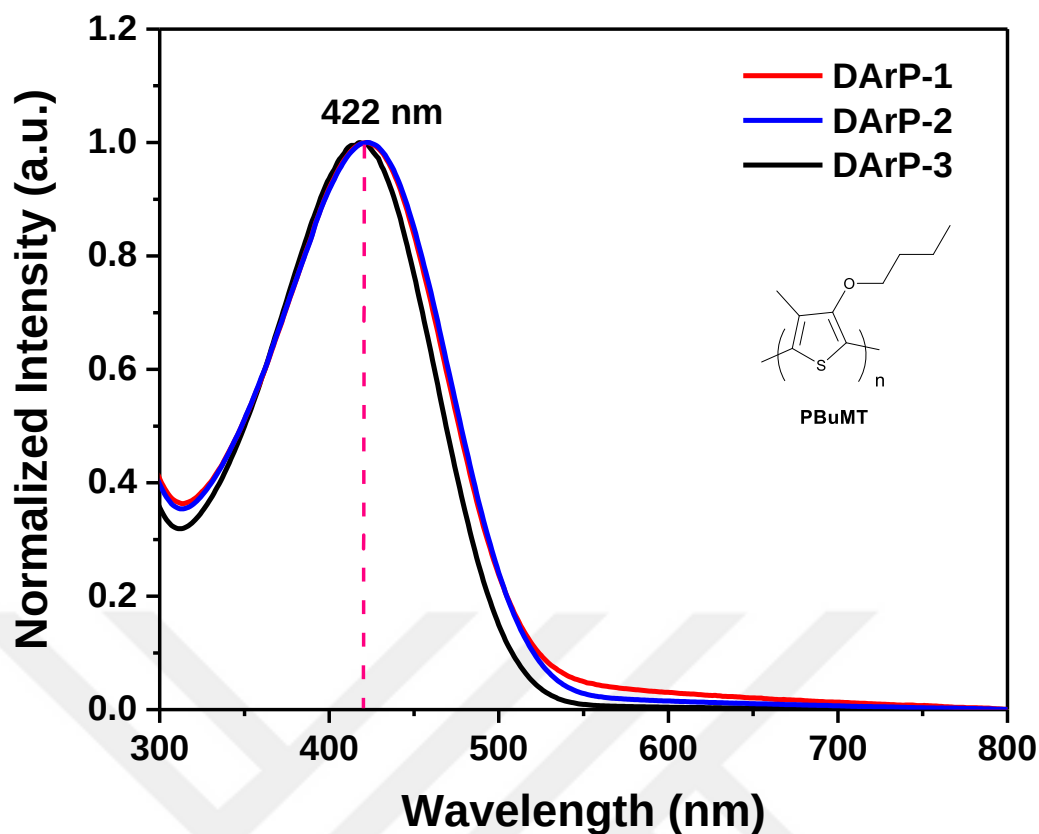


Figure 3.23. Absorption spectrum of PBUtMT via three different DArP protocols.

NIR emissive donor-acceptor based P4, and P5 polymers were tried to synthesize with successfully tested DArP protocols. After the synthesis, the resulting precipitants were analyzed via UV-visible spectroscopy. Figure 3.24 displays the absorption spectra of the resultant precipitants derived from P4 polymers. As seen in Figure, the spectra have a maximum wavelength of 239, 359, and 524 nm for nearly all DArP protocols. Because these peaks are part of the BBT acceptor unit, these tests were not able to confirm P4.⁸³ Then, the same trial was repeated for the P5 polymers, which have different donor sources as a DiBuT, but the results showed failed polymerization. After the synthesis of P5, no polymerization findings were seen like in P4. The electronic properties of the acceptor unit may cause the situation.

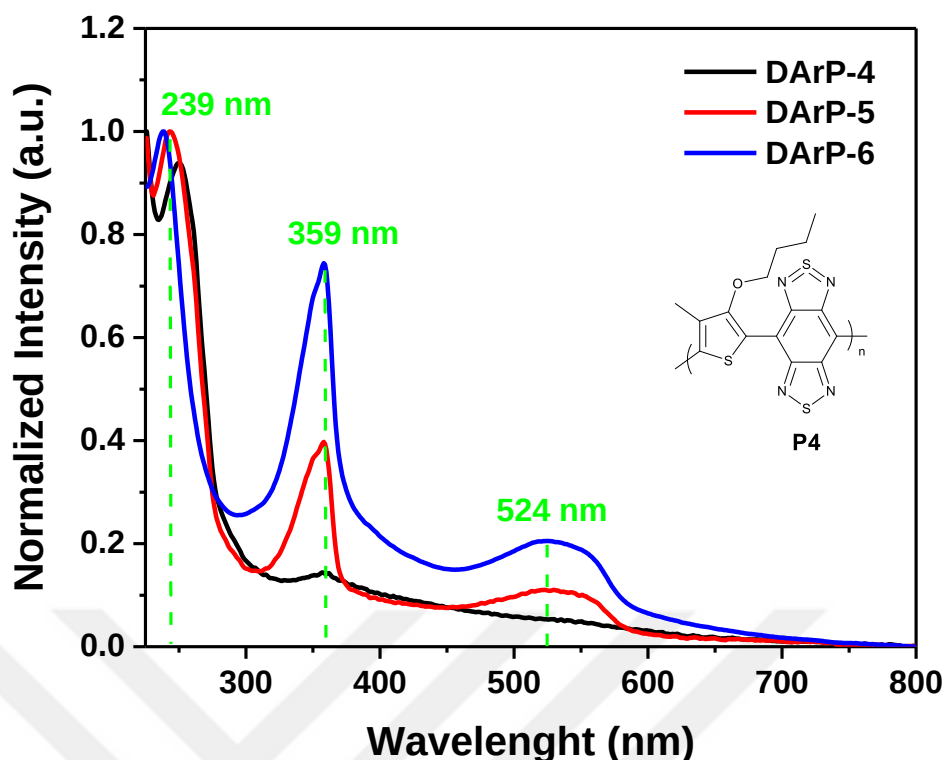


Figure 3.24. Absorption spectra for the P4 via three different DArP protocols.

3.2.3. Stille Polymerization Products

NIR emissive conjugated polymers (P6–10) were synthesized via Stille polymerization. Accordingly, five different donor-acceptor based conjugated polymers were prepared as P6–10. In this polymerization, two donor molecules were used: DiBuT and EDOT, with the acceptor unit BBT. The first step of the Stille polymerization includes the stannylation of the thiophene derivatives. Accordingly, EDOT was stannylated on the 2, and 5 positions of the thiophene. Therefore, two-sided stannylated EDOT (2,5-bis(tributylstannyl)-3,4-ethylenedioxythiophene) was synthesized. The compound was characterized via ^1H NMR spectroscopy. As seen in Figure 3.25, two-sided stannylated EDOT was structurally confirmed. ^1H NMR (400 MHz, CDCl_3) δ 4.12 (s, 4H), 1.61 – 1.54 (m, 12H), 1.39 – 1.30 (m, 12H), 1.12 – 1.08 (m, 12H), 0.90 (t, $J = 7.3$ Hz, 18H).

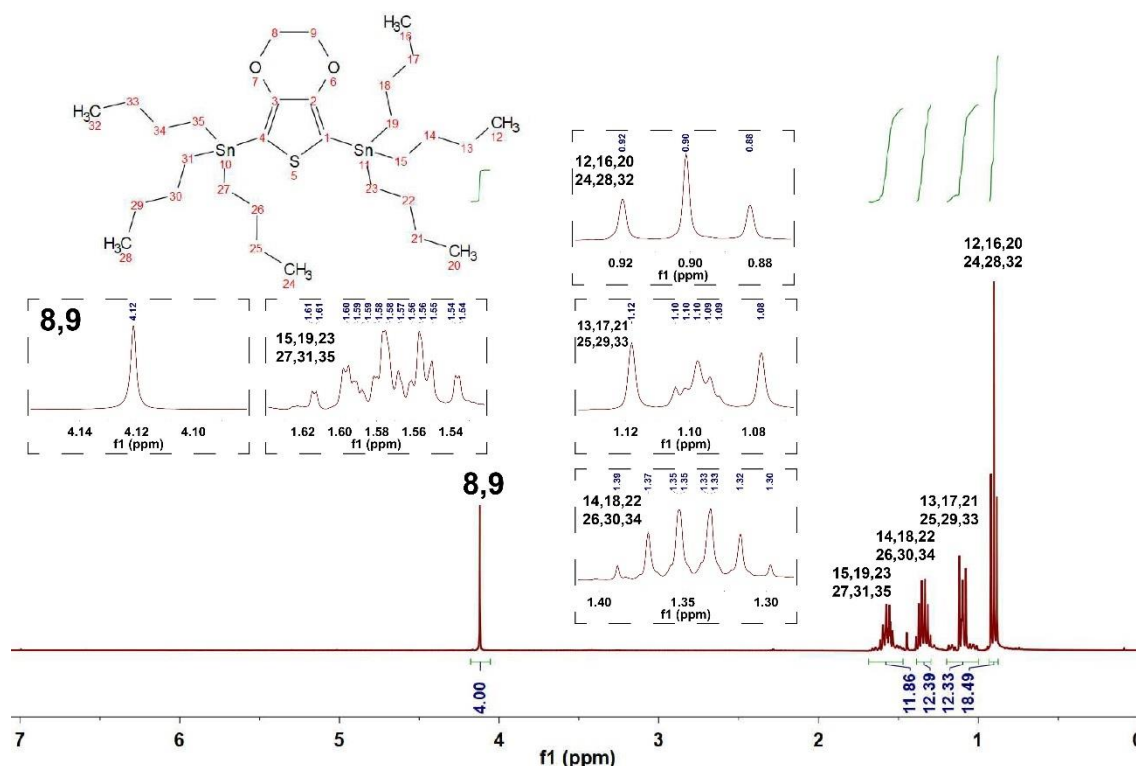


Figure 3.25. ^1H NMR spectrum of the two-sided stannylated EDOT.

Secondly, DiBuT was stannylated on the 2, and 5 positions of the thiophene. Therefore, two-sided stannylated DiBuT (2,5-bis(tributylstannyl)-3,4-dibutoxythiophene) was synthesized. The compound was characterized via ^1H NMR spectroscopy (Figure 3.26). As a result of the analysis, two-sided stannylated DiBuT was structurally confirmed. ^1H NMR (400 MHz, CDCl_3) δ 3.90 (t, J = 6.8 Hz, 4H), 1.73 – 1.65 (m, 4H), 1.61 – 1.49 (m, 12H), 1.49 – 1.38 (m, 4H), 1.37 – 1.27 (m, 12H), 1.13 – 1.02 (m, 12H), 0.95 (t, J = 7.4 Hz, 6H), 0.88 (t, J = 7.3 Hz, 18H).

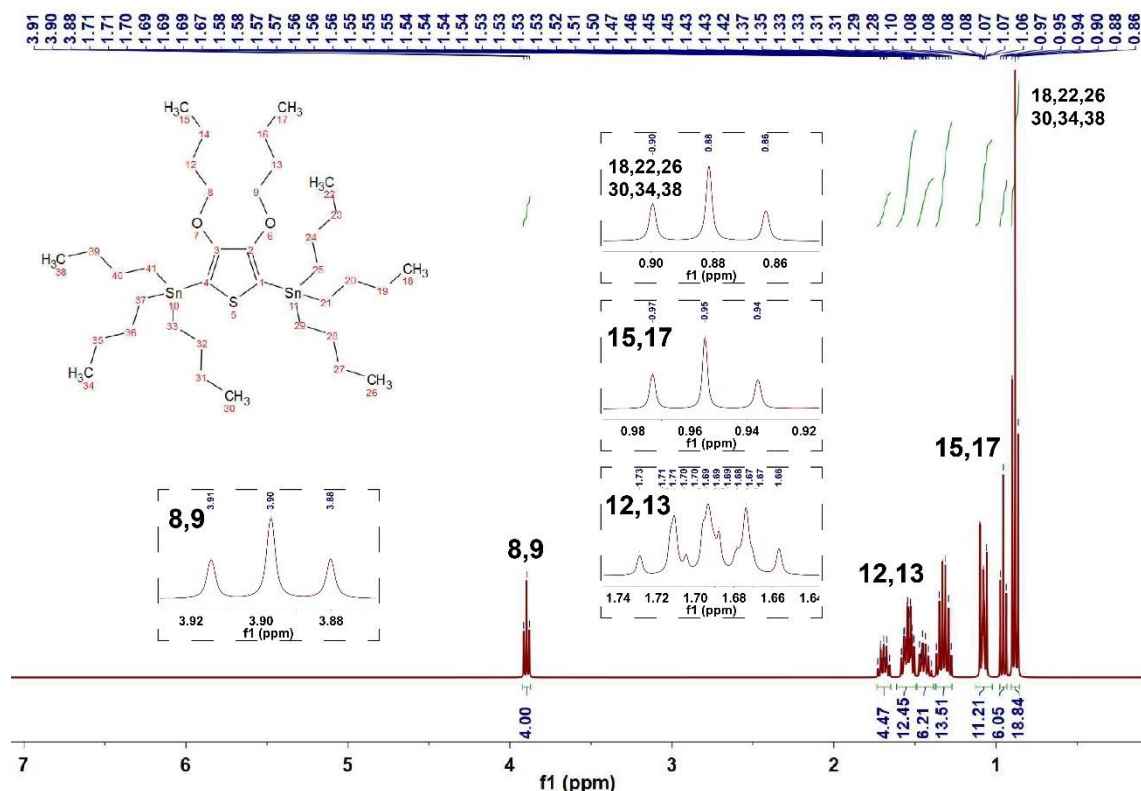


Figure 3.26. ^1H NMR spectrum of the two-sided stannylated DiBuT.

After the stannylation of the donor compounds, Stille polymerization was performed with the different molar ratios of the reactants. The polymerization reactions provided the five NIR emissive conjugated polymers as P6-10. Firstly, P6 was characterized to determine the photo-physical properties of the polymer by UV-visible and fluorescence spectroscopy. The Figure 3.27 shows the absorption and emission λ_{max} values of 755 nm and 881 nm, respectively. The results demonstrate that the P6 polymer has a broad absorption spectrum between 500 nm, and 1000 nm with a λ_{max} of 755 nm. Then, the P6 polymer was excited at 700 nm, giving a broad emission spectrum between 750 nm, and 1100 nm with a λ_{max} of 881 nm. As a result of the analysis, P6 has a strong emission in the NIR region.

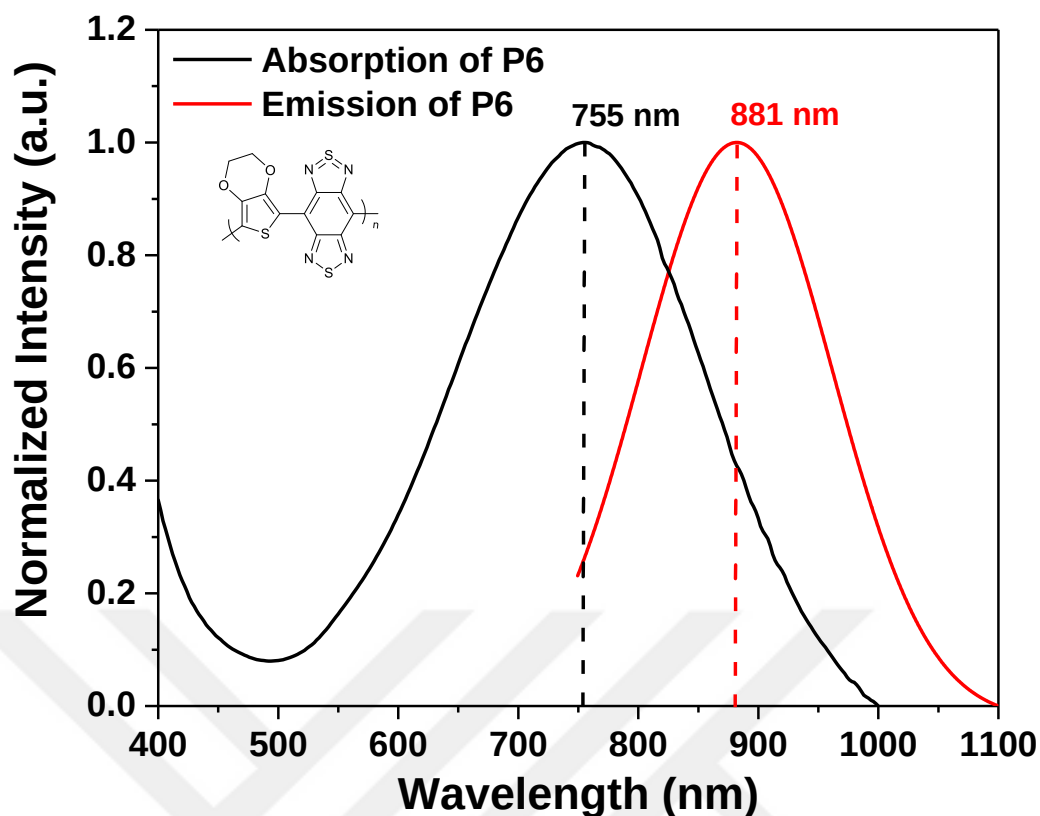


Figure 3.27. Absorption and emission spectrum of P6 in chloroform (excitation wavelength: 700 nm).

P7 was characterized to determine the photo-physical properties of the polymer by UV-visible and fluorescence spectroscopy. The Figure 3.28 shows the absorption and emission λ_{max} values of 741 nm and 883 nm, respectively. The results demonstrate that the P7 polymer has a broad absorption spectrum between 500 nm, and 1000 nm with a λ_{max} of 741 nm. Then, the P7 polymer was excited at 700 nm, giving a broad emission spectrum between 750 nm, and 1100 nm with a λ_{max} of 883 nm. As a result of the analysis, P7 has a strong emission in the NIR region.

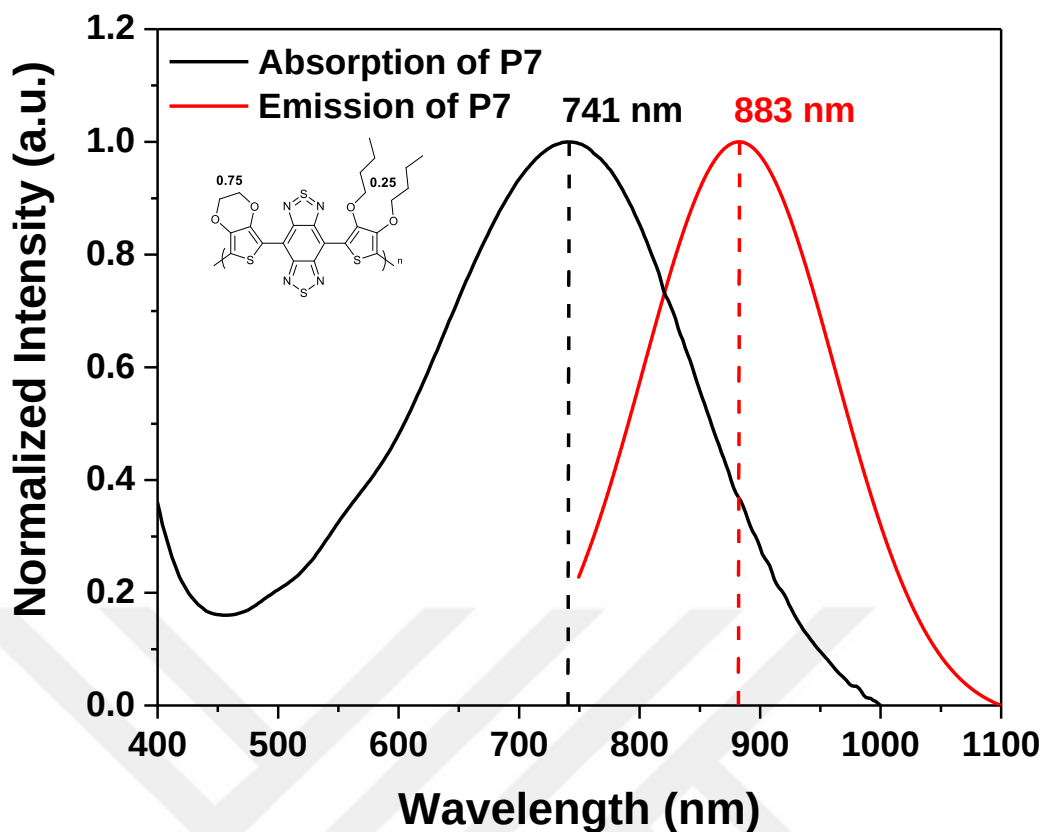


Figure 3.28. Absorption and emission spectrum of P7 in chloroform (excitation wavelength: 700 nm).

P8 was characterized to determine the photo-physical properties of the polymer by UV-visible and fluorescence spectroscopy. The Figure 3.29 shows the absorption and emission λ_{max} values of 705 nm and 875 nm, respectively. The results demonstrate that the P8 polymer has a broad absorption spectrum between 450 nm, and 1000 nm with a λ_{max} of 705 nm. However, an extra peak was seen at approximately 530 nm which was attributed to the BBT. The efficiency of the polymerization was slowly decreased with an increasing mol ratio of two-sided stannylated DiBuT because of the butoxy group of the DiBuT. Therefore, BBT may remain in the resulting product. Then, the P8 polymer was excited at 700 nm, giving a broad emission spectrum between 750 nm, and 1100 nm with a λ_{max} of 875 nm. As a result of the analysis, P8 has a strong emission in the NIR region.

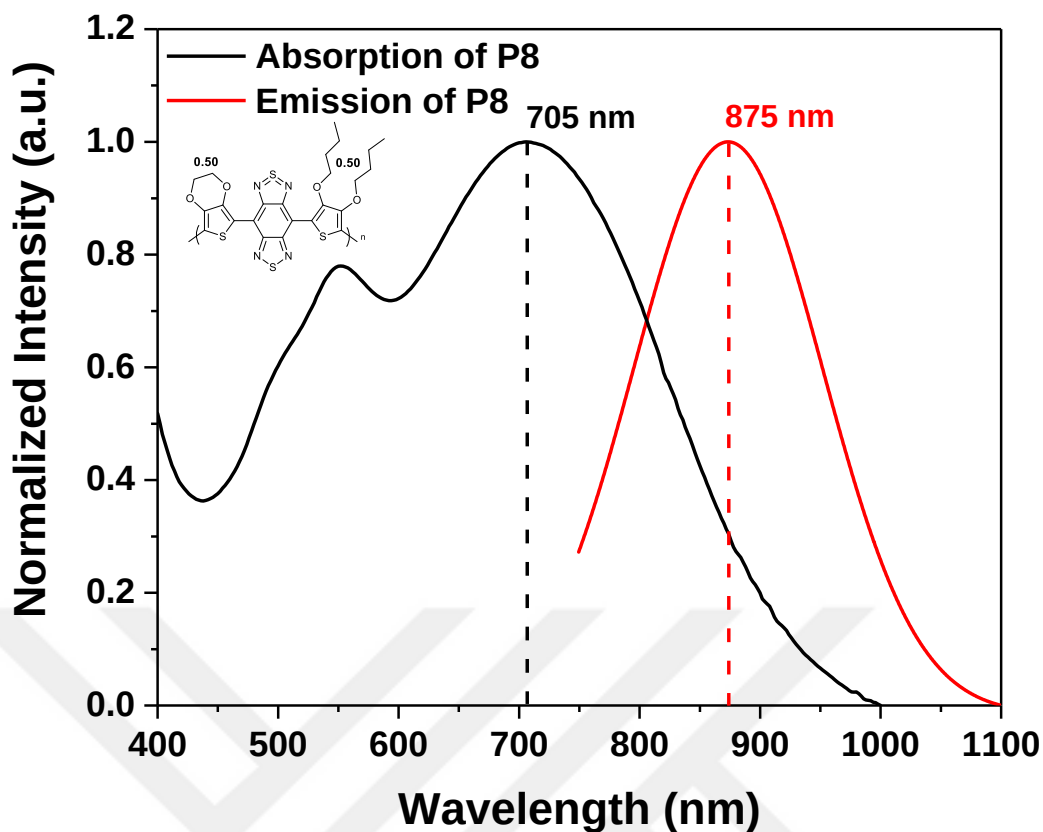


Figure 3.29. Absorption and emission spectrum of P8 in chloroform (excitation wavelength: 700 nm).

P9 was characterized to determine the photo-physical properties of the polymer by UV-visible and fluorescence spectroscopy. The Figure 3.30 shows the absorption and emission λ_{max} values of 680 nm and 810 nm, respectively. The results demonstrate that the P9 polymer has a broad absorption spectrum between 400 nm, and 1000 nm with a λ_{max} of 680 nm. However, an extra peak was seen at 543 nm which was attributed to the BBT. The efficiency of the polymerization was slowly decreased with an increasing mol ratio of two-sided stannylated DiBuT because of the butoxy group of the DiBuT. Therefore, BBT remained in the resulting product. Then, the P9 polymer was excited at 650 nm, giving a broad emission spectrum between 700 nm, and 1000 nm with a λ_{max} of 810 nm. As a result of the analysis, P9 has a strong emission in the NIR region.

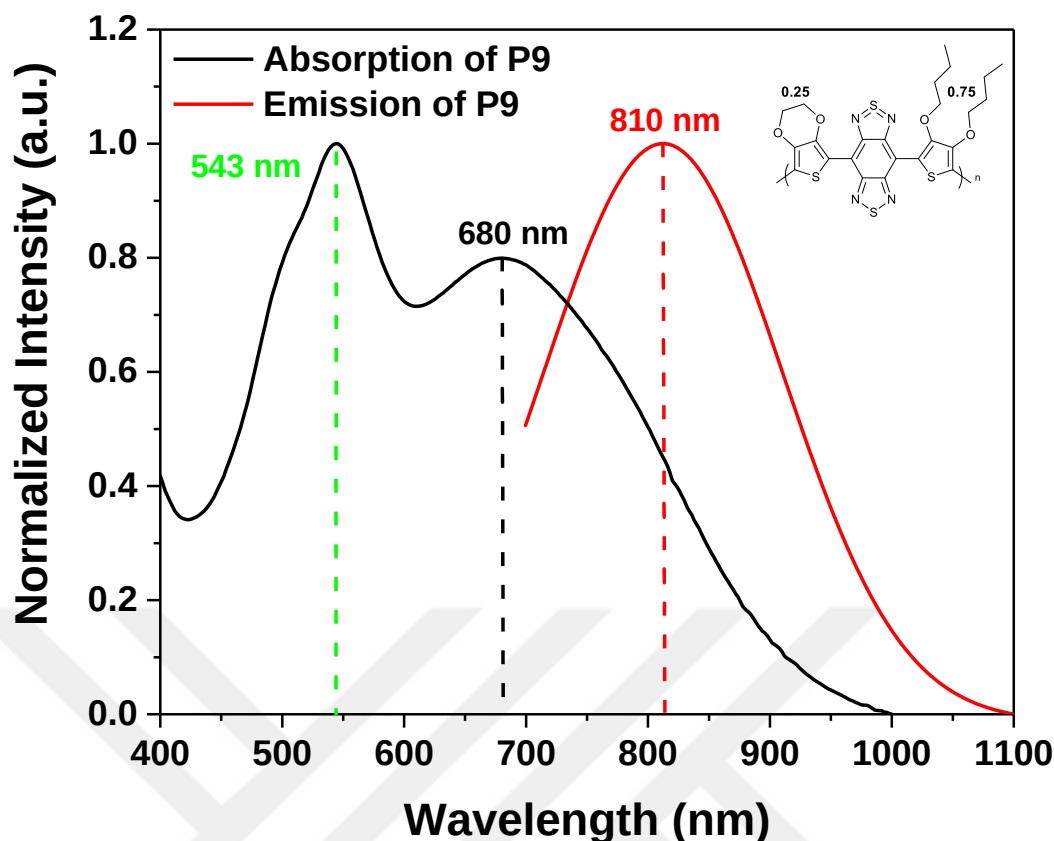


Figure 3.30. Absorption and emission spectrum of P9 in chloroform (excitation wavelength: 650 nm).

P10 was characterized to determine the photo-physical properties of the polymer by UV-visible and fluorescence spectroscopy. Figure 3.31 shows the absorption and emission λ_{max} values of 543 nm and 810 nm, respectively. The results demonstrate that the P10 polymer has an absorption spectrum between 400 nm, and 850 nm with a wavelength of 630 nm. The peak at 630 nm was not seen because of the lower efficiency of the polymerization. However, an extra peak was seen at 543 nm which was attributed to the BBT. The efficiency of the polymerization was decreased with a two-sided stannylated DiBuT because of the butoxy group of the DiBuT. Therefore, BBT remained in the resulting product. Then, the P10 polymer was excited at 630 nm, giving an emission spectrum between 650 nm, and 900 nm with a λ_{max} of 775 nm. As a result of the analysis, P10 has an emission in the NIR region.

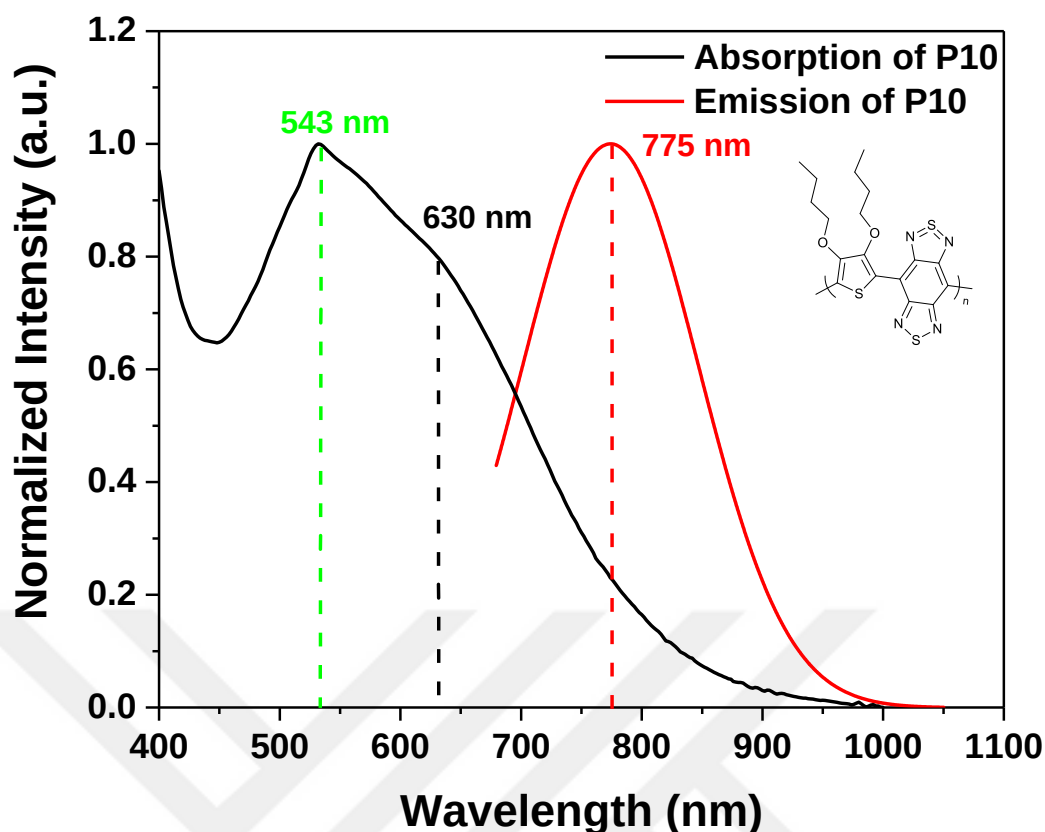


Figure 3.31. Absorption and emission spectrum of P10 in chloroform (excitation wavelength: 630 nm).

After the individual examination of each Stille polymerization product, the trend was compared in the optic properties of all Stille polymerization products (P6–P10). Accordingly, Figure 3.32 shows the absorption spectra for the polymers. The figure illustrates that as the two-sided stannylated EDOT mol ratios increase in the contents of the Stille polymerization protocols, the maximum wavelength tends to be higher than other wavelengths. The absorption λ_{max} was characterized for the polymers P6–10 as 755 nm, 741 nm, 705 nm, 680 nm, and 630 nm, respectively. The trend was attributed to the butoxy group of the DiBuT because the aliphatic group decreased the polymerization efficiency. On the other hand, EDOT does not have a steric hindrance as compared to DiBuT. That's why, if the EDOT content of the NIR-emissive polymers increases, absorption λ_{max} values also increase.

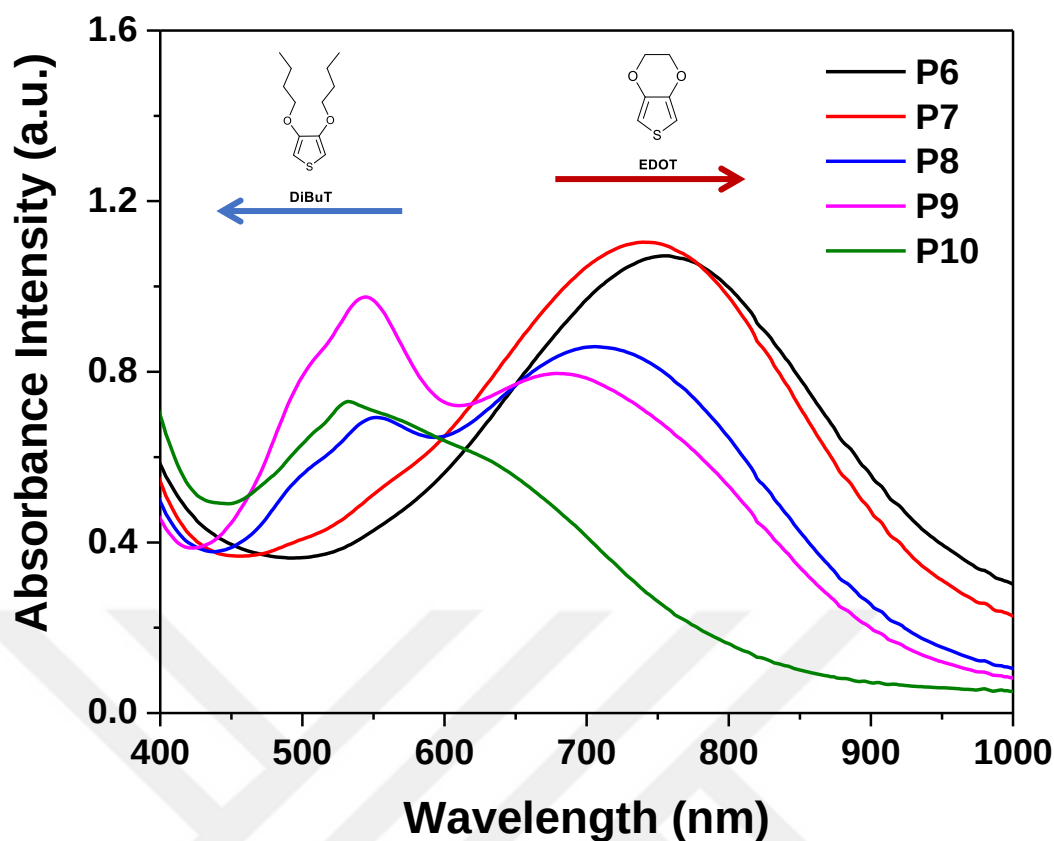


Figure 3.32. Absorbance spectra for all Stille polymerization products in chloroform.

Then, the trend was investigated in emission spectra of the Stille polymerization products (P6-10) (Figure 3.33). The figure illustrates that as the two-sided stannylated EDOT mol ratios increase in the contents of the Stille polymerization protocols, the maximum wavelength tends to be higher than other wavelengths. The emission λ_{max} was characterized for the polymers P6-10 as 881 nm, 883 nm, 875 nm, 810 nm, and 775 nm, respectively. The trend was attributed to the butoxy group of the DiBuT because the aliphatic group decreased the polymerization efficiency. On the other hand, EDOT does not have a steric hindrance as compared to DiBuT. That's why, if the EDOT content of the NIR emissive polymers increases, emission λ_{max} values also increase.

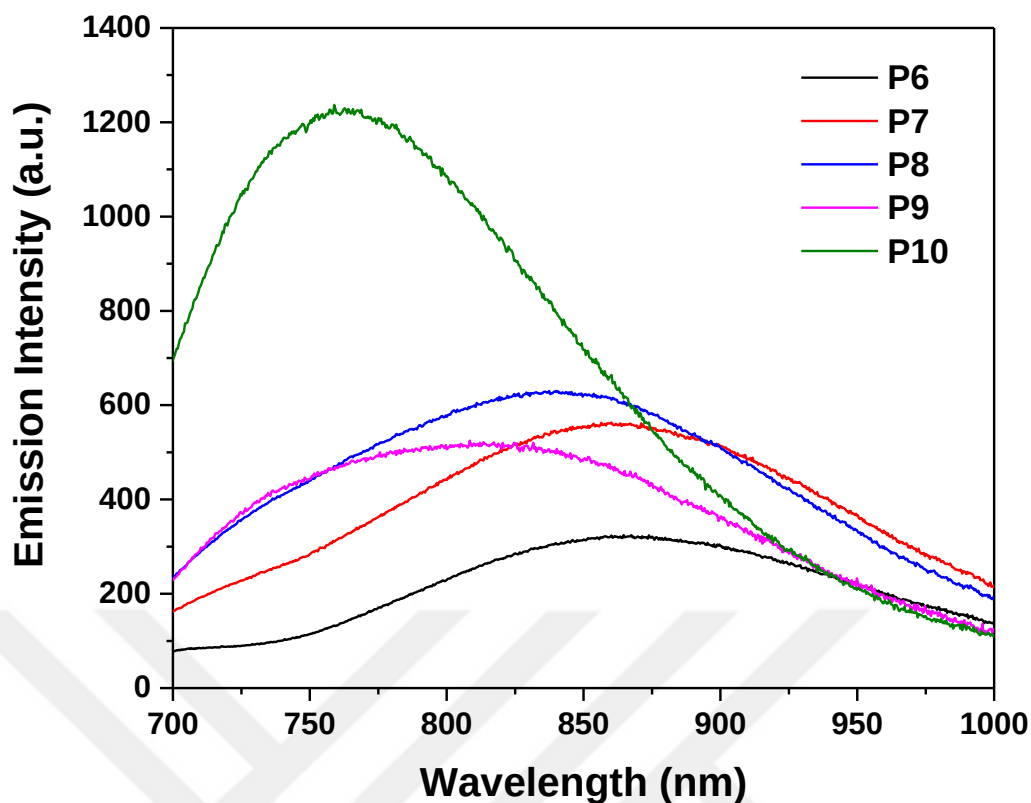


Figure 3.33. Emission spectra for all Stille polymerization products in chloroform (excitation wavelength: 650 nm).

The Stille polymerization products (P6–10) were photographed under long-wave UV light. As seen in Figure 3.34, visibility of the color, which also means emission capability in the visible region, increases with increasing content of DiBuT in Stille polymers. The photographs confirmed a decrease in polymerization efficiency with an increasing DiBuT content in Stille polymers. For example, P6 and P7 NIR emissive conjugated polymers have no visibility, which means the polymers have emission in the NIR region.

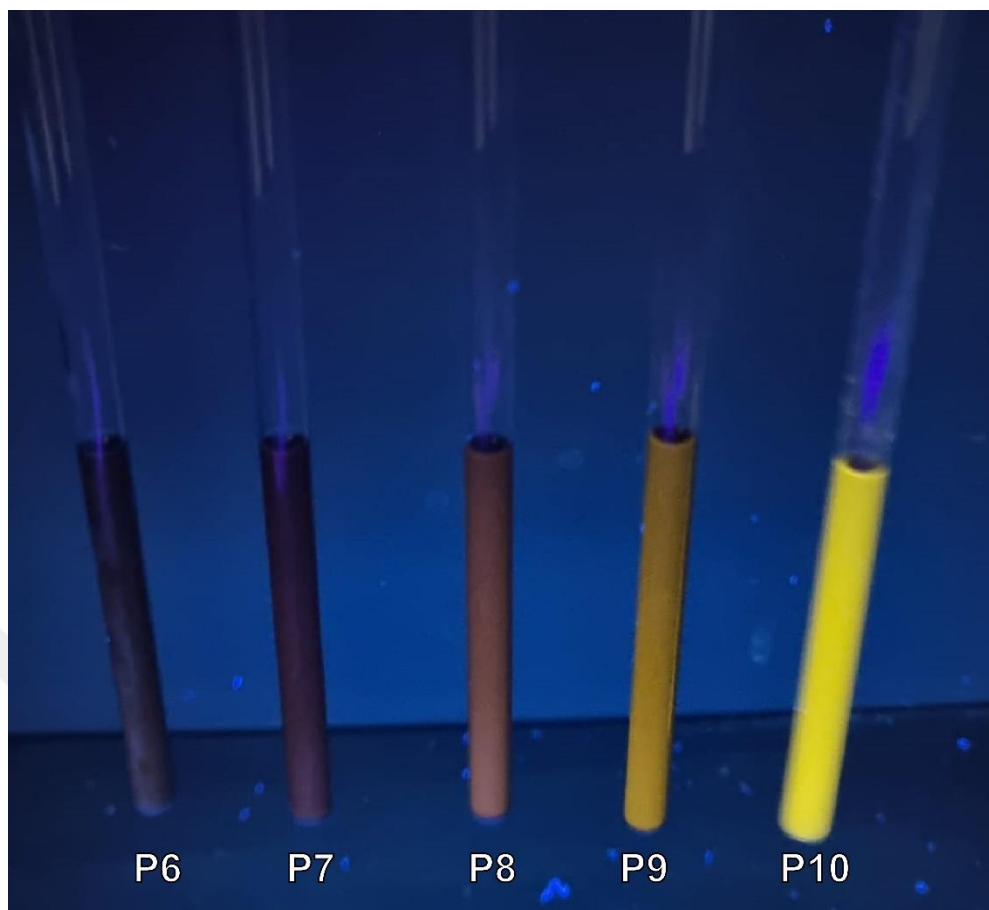


Figure 3.34. Photograph of the Stille polymerization products (P6–10) in chloroform under long wave UV light.

3.3. Characterization of Pdots

In this step, a P1 NIR emissive conjugated polymer, which was synthesized via oxidative polymerization, was used to prepare the Pdots with sonication-assisted emulsification. The P1 NIR emissive polymer was selected as a model polymer for bioimaging applications and its further characterization as a Pdot (Figure 3.35).

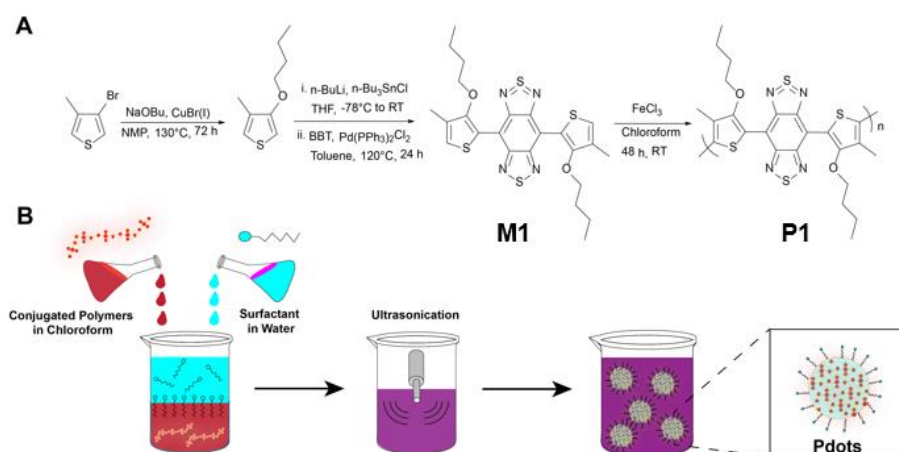


Figure 3.35. (A) Synthesis protocol of NIR emissive P1, (B) schematic representation of the preparation of Pdots.

After the preparation of Pdot, it was optically characterized, with absorption and emission maximum wavelengths of 650 nm and 861 nm in water, respectively (Figure 3.36). The Pdot emission spectra exhibit an emission peak ranging from 700 to 1000 nm, which belongs to the near-infrared (NIR) region. The Stokes shift, which was determined to be 211 nm, effectively reduces Pdot's self-absorption. As previously demonstrated, Pdots composed of D-A-D type polymers likewise exhibited significant Stokes shifts and negligible self-absorbance. The primary benefit of NIR emissive Pdots is clearly evident in their minimal interference with the emission signal.²⁷

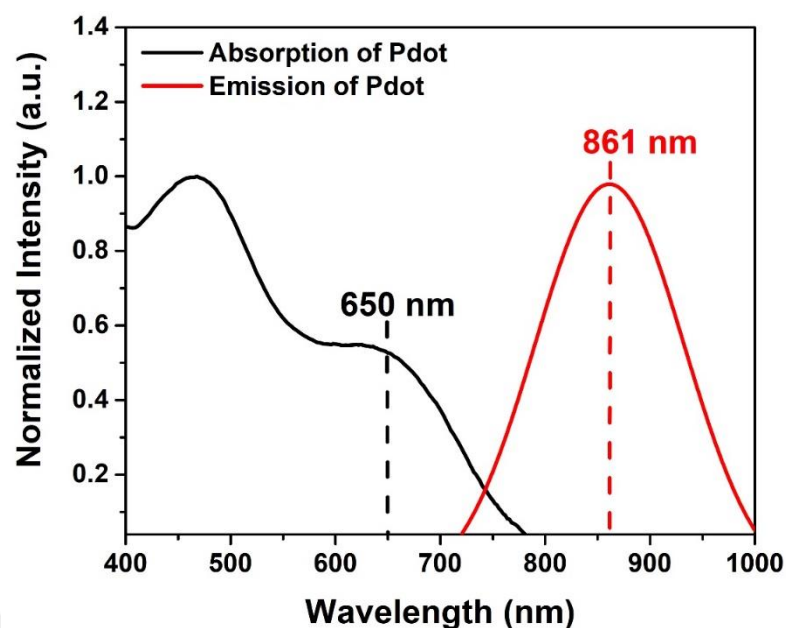


Figure 3.36. Absorption and emission spectra of NIR emissive Pdot in water.

The photostability of the NIR emissive Pdot was tested (Figure 3.37). The Pdot sample was placed in a glass vial. Also, the sample was positioned 15 cm away from the light source (red LED, 620–630 nm).⁸⁴ The incident radiation intensity was calculated at 3.3 mW/cm². Absorbance measurements were performed for 5 hours at 30-minute time intervals, and the degradation rate was calculated from the integrated rate law equation ($\ln(A_t/A_0) = -k_{\text{degt}}$). Then, the half-life of the NIR emissive Pdot was calculated as 135.9 h.

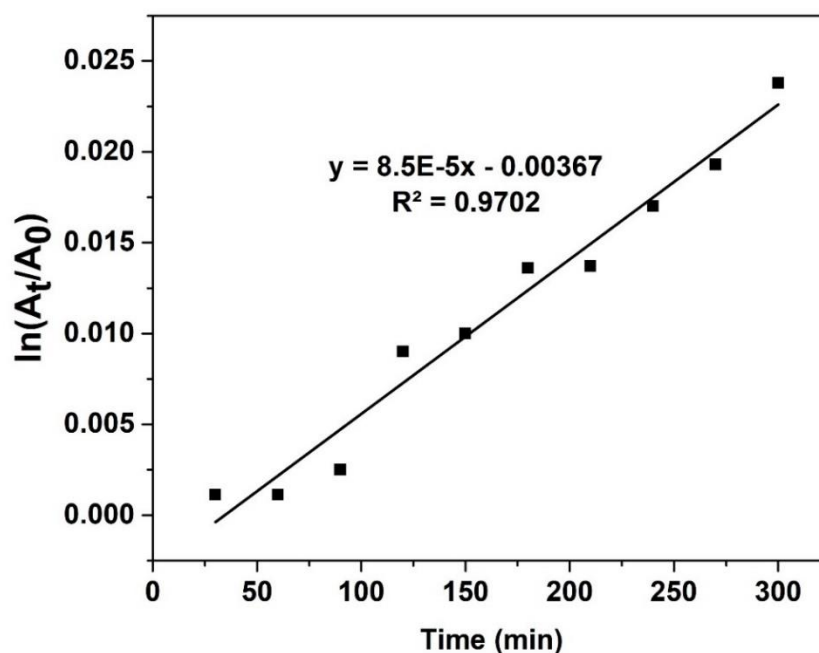


Figure 3.37. The graph of $\ln(A_t/A_0)$ versus time for the NIR emissive Pdot.

The zeta potential of CTAB micelles was evaluated in both the presence and absence of nonionic polymer. After measuring the final zeta potentials for several trials, it was determined that they were 68.8 ± 9.3 mV and 51.8 ± 9.5 mV in the presence and absence of nonionic polymer. The overlap percentage of 27.15% was obtained from the computed area under the Gaussian curves, and 72% of the nonionic polymer containing CTAB micelles was identified (Figure 3.38).

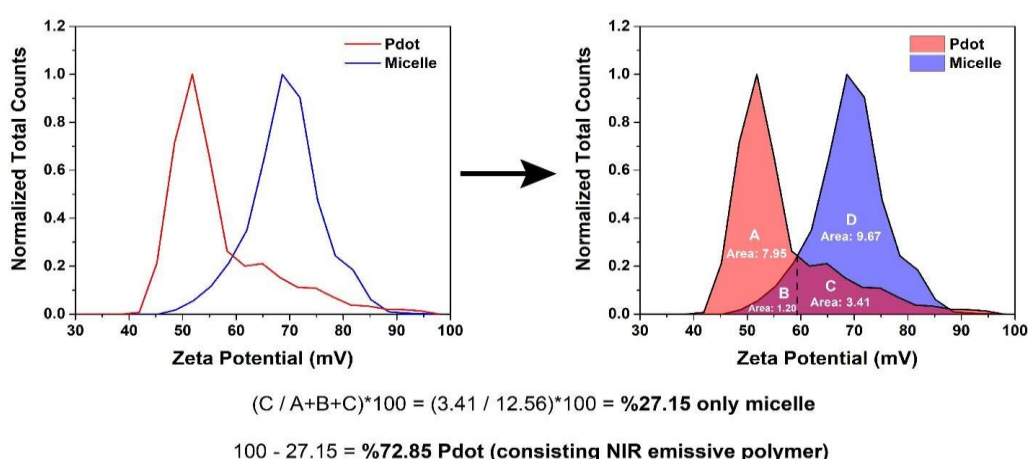


Figure 3.38. Analysis of Pdots containing NIR emissive polymers in solution with size measurements for pure CTAB micelles and NIR emissive Pdot.

Furthermore, an experiment was conducted to ascertain the number-averaged of pure CTAB nanoparticles (NPs) and NIR emissive Pdots in the presence and absence of nonionic polymer. All other parameters remained constant for both scenarios. After measuring the final diameters for several trials, it was estimated that they were roughly 5 nm and 12 nm, respectively, in the presence and absence of nonionic polymer (Figure 3.39). CTAB NPs have an average hydrodynamic radius of approximately 2 to 5 nm according to the literature. For this reason, the CTAB NPs were assigned for the ~5 nm size finding. However, for NIR emissive Pdot, the average hydrodynamic radius was found to be close to ~12 nm. As understood, most of the CTAB micelles which were in NIR emissive Pdot solution, include the NIR emissive polymer.

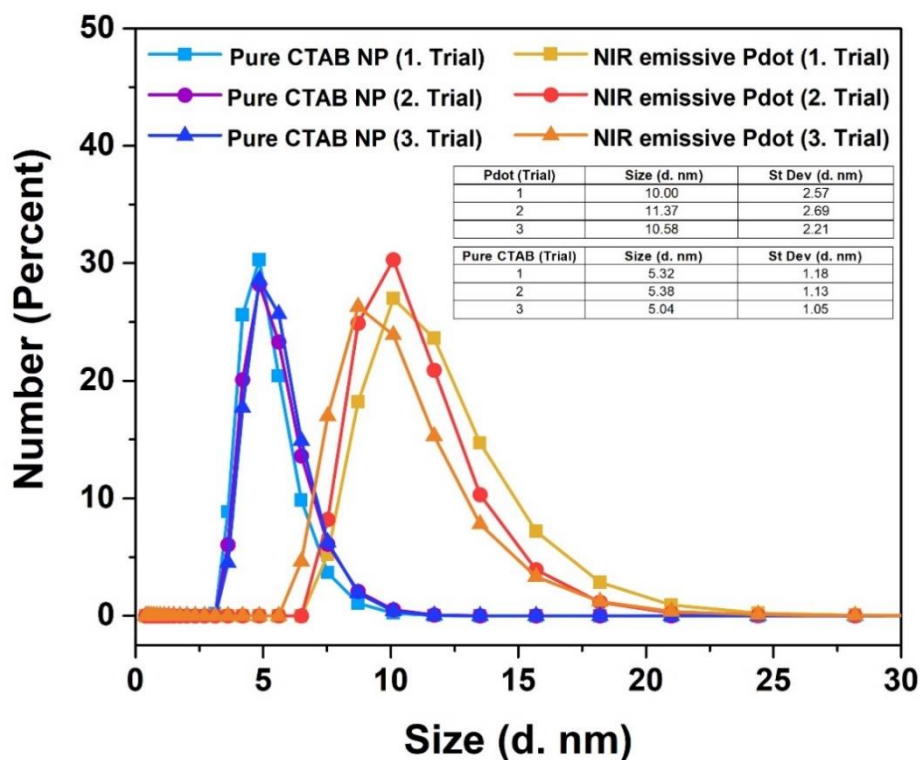


Figure 3.39. Analysis of pure CTAB nanoparticles (NPs) and the Pdots containing NIR emissive polymers in solution with the number-averaged size measurements.

The size, zeta potential (ZP), mobility (Mob), and conductivity (Cond) values of Pdots at various time points (1., 15., and 30. Day) with changing pH (between 5.5-7.5) have been studied and reported at Table 3.3. pH has a minor impact on the size and zeta potential of Pdots, but it does not result in instability, as in Table 3.3 and Figure 3.40

demonstrate. Specifically, zeta potential values greater than 50 mV across all pH ranges validate colloidal stability.

Table 3.3. Size, zeta potential (ZP), mobility (Mob), and conductivity (Cond) values for the NIR emissive Pdots at different pH values and time points (5.5–7.5).

Time (Day)	pH	Size (nm)	ZP (mV)	Mob ($\mu\text{mcm/Vs}$)	Cond (mS/cm)
1. Day	5.5	12.5 $\pm$ 2.6	54.5 $\pm$ 1.97	4.30 $\pm$ 0.15	0.320
	6.0	11.5 $\pm$ 3.1	53.9 $\pm$ 3.10	4.22 $\pm$ 0.24	0.152
	6.5	22.3 $\pm$ 8.6	51.3 $\pm$ 3.56	4.02 $\pm$ 0.28	0.783
	7.0	12.7 $\pm$ 3.6	53.6 $\pm$ 1.11	4.20 $\pm$ 0.09	0.158
	7.5	11.5 $\pm$ 3.2	58.4 $\pm$ 2.43	4.58 $\pm$ 0.19	0.162
15. day	5.5	13.8 $\pm$ 4.8	55.7 $\pm$ 4.88	4.37 $\pm$ 0.38	0.308
	6.0	11.6 $\pm$ 3.2	53.8 $\pm$ 0.66	4.22 $\pm$ 0.05	0.165
	6.5	24.4 $\pm$ 8.4	53.9 $\pm$ 3.40	4.23 $\pm$ 0.27	0.734
	7.0	13.5 $\pm$ 3.6	52.2 $\pm$ 1.01	4.09 $\pm$ 0.08	0.176
	7.5	12.1 $\pm$ 3.5	59.2 $\pm$ 1.69	4.64 $\pm$ 0.13	0.173
30. Day	5.5	16.4 $\pm$ 3.4	52.4 $\pm$ 1.50	4.11 $\pm$ 0.12	0.296
	6.0	13.0 $\pm$ 3.1	55.1 $\pm$ 0.09	4.32 $\pm$ 0.07	0.171
	6.5	24.1 $\pm$ 8.4	56.1 $\pm$ 0.40	4.40 $\pm$ 0.03	0.170
	7.0	13.6 $\pm$ 3.9	53.6 $\pm$ 3.30	4.20 $\pm$ 0.26	0.733
	7.5	12.4 $\pm$ 3.3	58.3 $\pm$ 0.14	4.57 $\pm$ 0.01	0.177

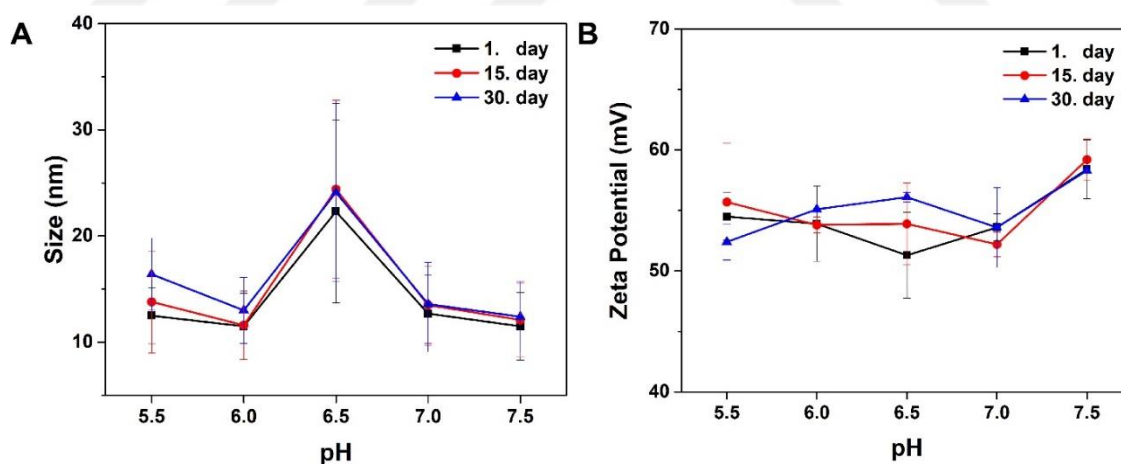


Figure 3.40. (A) Size values at different time points and pH range (5.5–7.5) graph for the NIR emissive Pdot; (B) Zeta potential values at different time points and pH range (5.5–7.5) graph for the NIR emissive Pdot.

The scanning electron microscopy (SEM) method was used to investigate the morphological characteristics of Pdots. As depicted in the Figure 3.41, a spherical shape was observed in all Pdots.

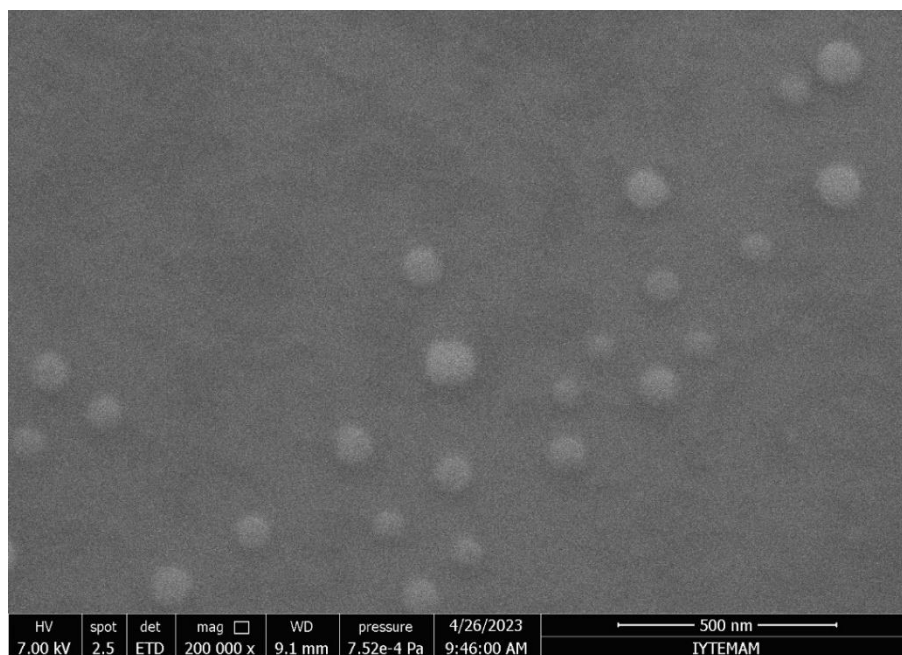


Figure 3.41. SEM image of NIR emissive Pdot.

In addition, AFM studies were carried out on the Pdot, indicating an average size of around 20 nm (Figure 3.42). The cross-sectional profiles of Pdots (Pdot 1-6) demonstrate the uniformity in both size and surface shape. Furthermore, the utilization of DLS allows for the determination of the average hydrodynamic radius of the Pdot, which measures 12 nm. The autocorrelation function depicted in Figure 3.43 (represented by a dotted line) demonstrates the monomodal relaxation, providing evidence for the homogeneous distribution of Pdot. The disparity in size between the DLS and AFM studies can be explained by the inclusion and elimination of solvents in these two investigations, leading to a substantial size alteration in the latter scenario mainly due to the flattening of soft objects.

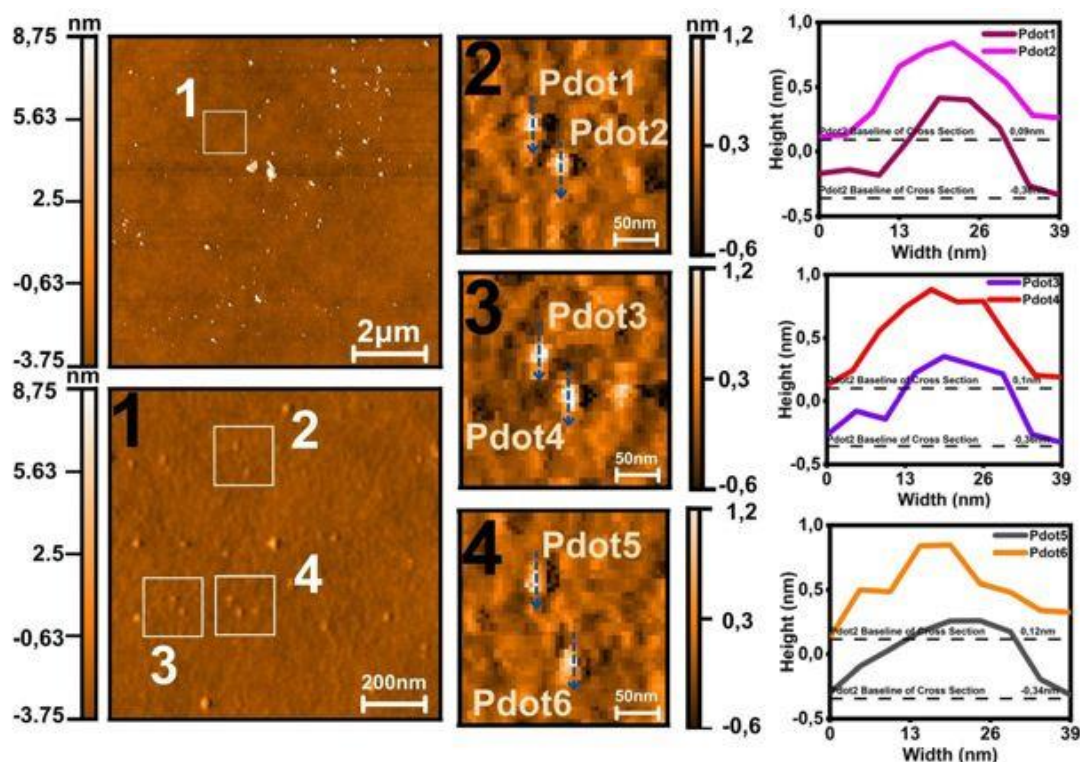


Figure 3.42. A) Topography images and cross-section analysis of the Pd dots (Pd dot1-6 bottom of this part) by AFM (scale bars for AFM images: 2 μ m, 200 nm, and 50 nm, respectively).

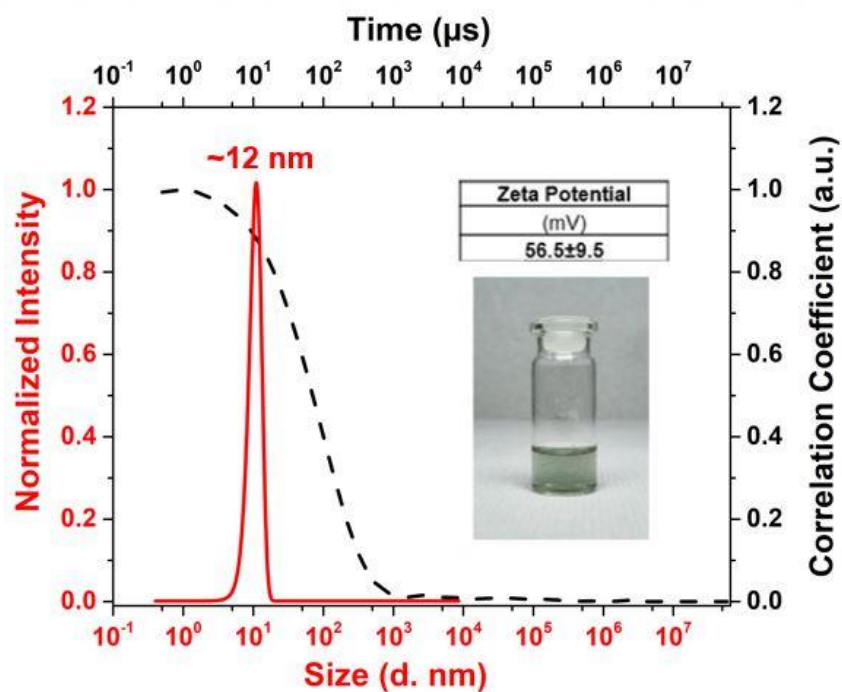


Figure 3.43. Size analysis, and auto-correlation graphs of NIR emissive Pd dot by DLS with a photograph of the Pd dot solution in water.

The zeta potential measurement of Pdot indicated that non-ionic Pdots were effectively stabilized in water as a result of their surface charge (Zeta Potential = 56.5 ± 9.5 mV, as shown in Table 3.4), which was provided by CTAB. The zeta potential, which is above 40 mV, has been assessed as a criterion for colloidal stability. Electrostatic force microscopy (EFM) in Figure 3.44 demonstrated the presence of a comparable charge distribution in the dry condition. The interaction between the Pdot-AFM tip is insignificant while the tip is at 0 V. However, when the tip is biased to 3V/-3V, electrostatic interactions (both attractive and repulsive forces) become the main determining factor. The phase angle was determined at 1.15° under 3V scanning and 2.00° under -3V scanning, indicating that the particles have a positive charge. This verifies that cationic Pdots were successfully stabilized and maintained their integrity during the drying process. This verifies that cationic Pdots were successfully stabilized and maintained their structural integrity during the drying process.

Table 3.4. Zeta potential measurement for NIR emissive Pdot.

	ZP (mV)	Mob ($\mu\text{mcm/Vs}$)	Cond (mS/cm)
NIR emissive Pdot	56.5 ± 9.5	4.43 ± 0.75	0.191

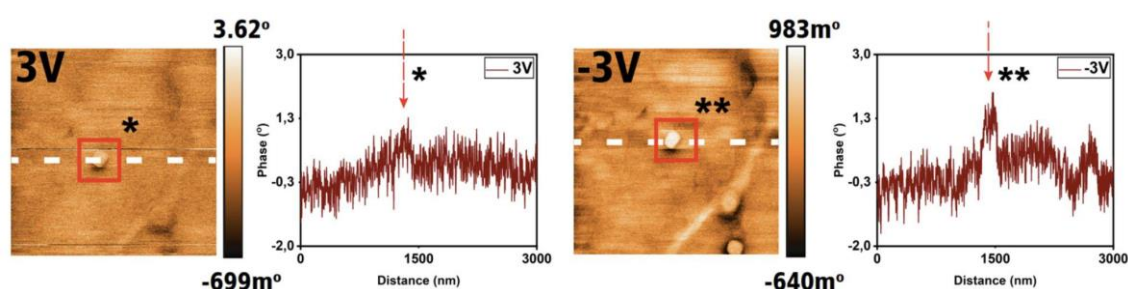


Figure 3.44. EFM topography images of NIR emissive Pdot at different voltage states and phase degree graphs respectively.

3.4. Optimization and Characterization of imaging parameters with Pdots in standard 2D cell culture models

The imaging characteristics of Pdot were optimized and characterized in a conventional 2D cell culture. The imaging component of standard 2D cell culture was optimized in this study, and imaging was carried out using the optimum parameters. The experiment utilized HeLa cells (human cervical epithelial adenocarcinoma cells), MDA-MB-231 cells (human breast adenocarcinoma cells that are estrogen-independent), MCF-7 cells (human breast adenocarcinoma cells that are estrogen-dependent), and SH-SY5Y cells (human cells) to produce 3D tumoroid models. 2D cell culture investigations and concentration optimization were carried out using neuroblastoma and PC-12 cancer cell lines. To increase the cell count, cells were cultivated in a flask and maintained in a conventional incubator (at a temperature of 37°C and with a CO₂ concentration of 5%) for 3 days. The cultivation medium used was DMEM, supplemented with L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (Pen/Strep). The cell lines cultivated in a 2D medium were removed using trypsin once they reached a growth rate of 80-90%. Following the inactivation of trypsin in the serum medium, centrifugation was utilized to eliminate the medium containing trypsin and collect the cells. Subsequently, individual cell lines were placed into 96 microplates at a cellular density of 5×10^4 cells per plate and left to incubate for 24 hours. Afterward, the NIR emissive Pdots were introduced to cancer cells that were placed in 96 microplates at a concentration of 60 μ M. After an incubation period of 24 hours, the cells were examined using a fluorescence microscope. To characterize Pdot-treated cells, the cell nucleus (blue) was stained with DAPI, and the regions where Pdot was present within the cell were compared to DAPI.

The Figure 3.45 displays fluorescence microscopy examinations of MCF-7, SH-SY5Y, PC-12, HeLa, and MDA-MB-23 cells that were treated with NIR emissive Pdot. Upon close examination of the DAPI, Pdot, and Calcein Green analyses, it was noted that the Pdot and Calcein Green stainings exhibited complete overlap. Still, the DAPI staining, which represents the cell nucleus, appeared to be dissociated. In particular, in HeLa cells, it is readily apparent that Pdot and DAPI dyes may be distinguished from one another, offering the benefit of precise cell size and shape identification.

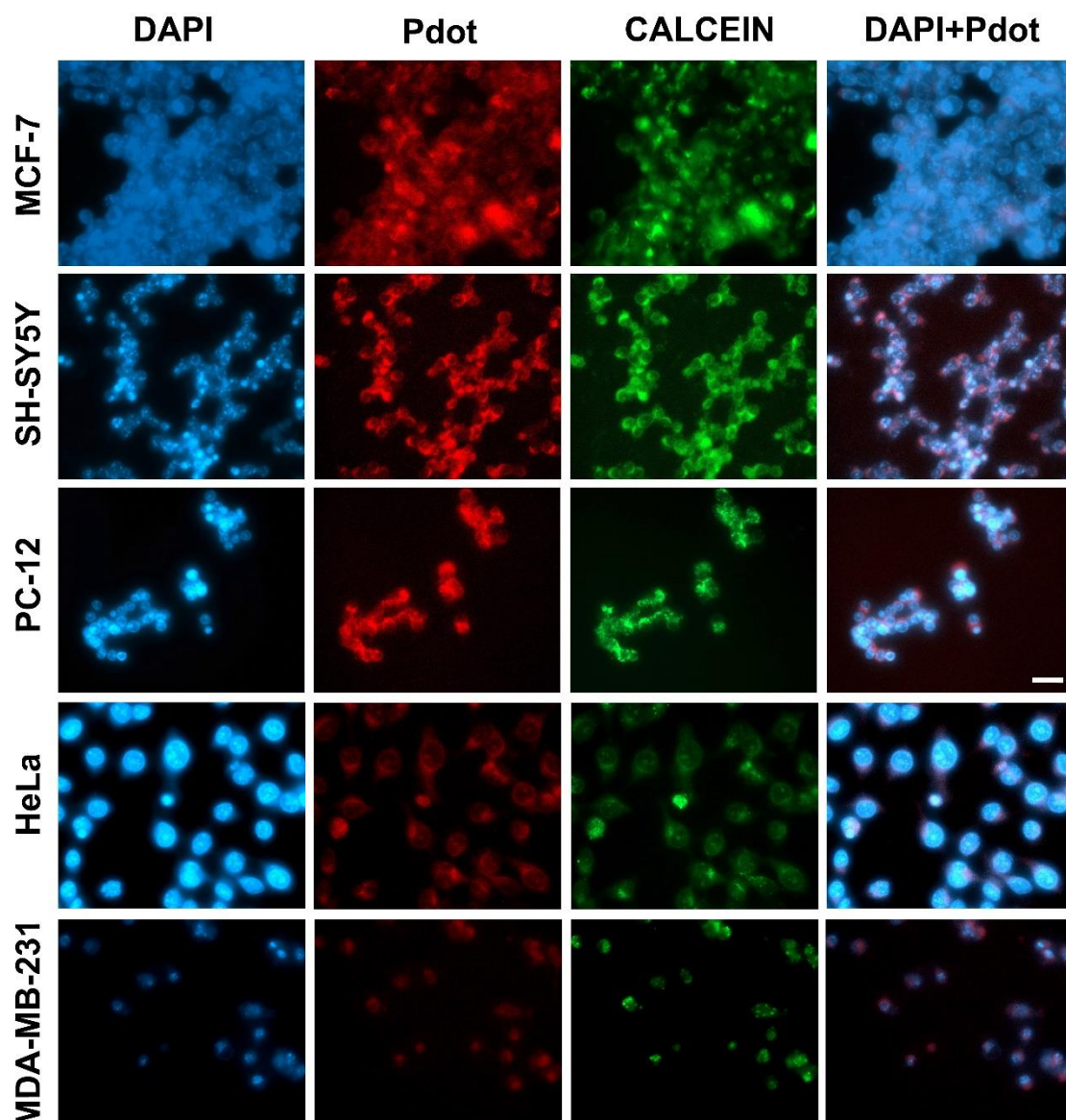


Figure 3.45. Fluorescence microscopy images of MCF-7, SH-SY5Y, PC-12, HeLa, and MDA-MB-23 cells, cultured with Pdot and labeled with DAPI and Calcein Green (blue: DAPI, red: Pdot, green: Calcein, scale bar: 20 μ m).

After the cells had been cultured with Pdots for 3, 5, and 7 days, a fluorescence microscope was used to evaluate the results. Images of Pdot, DAPI, and Calcein Green staining on days 3, 5, and 7 are displayed in Figure 3.46, Figure 3.47, and Figure 3.48. It has been observed that Pdots retained their photostability and that a longer incubation period had no effect on degradation. It was also shown that Pdots maintain an intense tendency to enter the nucleus even after extended cell culture. Additionally, the high

cell viability for a long time in the presence of Pdots is confirmed by Calcein Green staining.

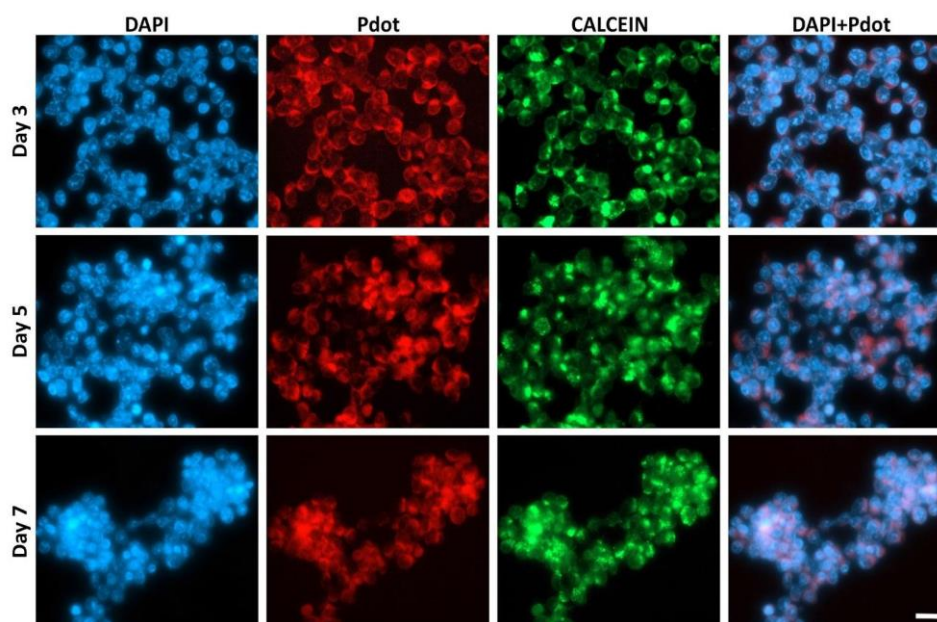


Figure 3.46. Fluorescence microscopy images of MCF-7 cells cultured with Pdot for 3, 5, and 7 days labeled with DAPI and Calcein Green (blue: DAPI, red: Pdot, green: Calcein, scale bar: 20 μ m).

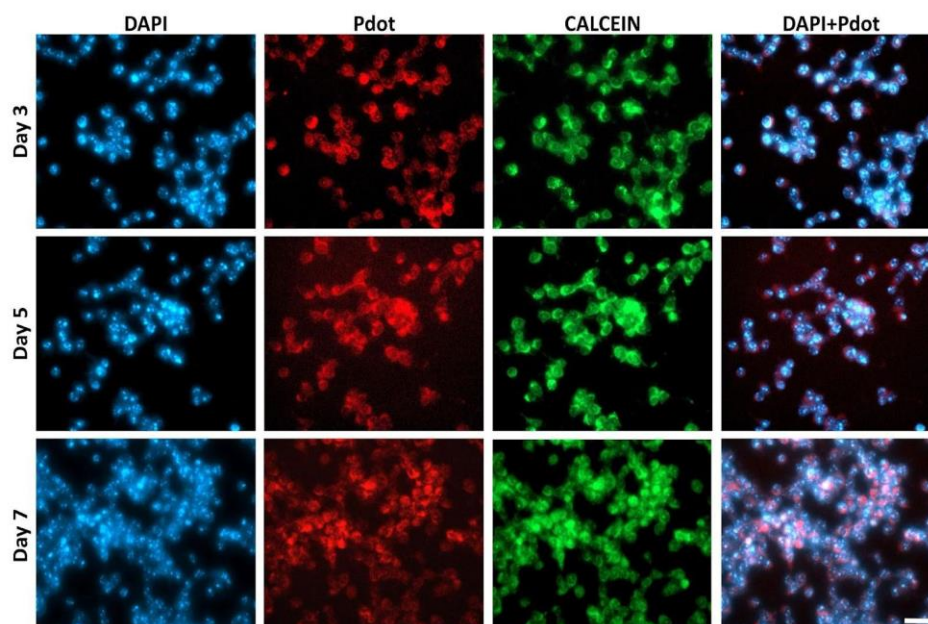


Figure 3.47. Fluorescence microscopy images of SH-SY5Y cells cultured with Pdot for 3, 5, and 7 days labeled with DAPI and Calcein Green (blue: DAPI, red: Pdot, green: Calcein, scale bar: 20 μ m).

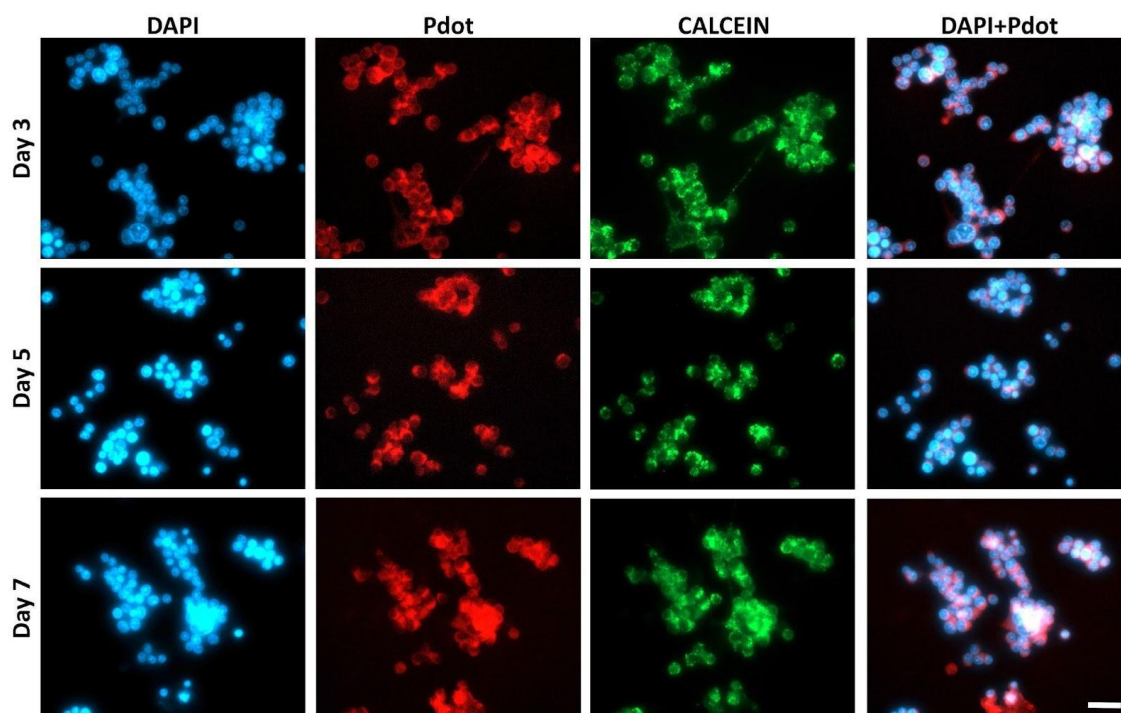


Figure 3.48. Fluorescence microscopy images of PC-12 cells cultured with Pdot for 3, 5, and 7 days labeled with DAPI and Calcein Green (blue: DAPI, red: Pdot, green: Calcein, scale bar: 20 μ m).

Also, the process of staining mitochondria was accomplished for all cell lines following incubation with Pdot, as shown in Figure 3.49. The Pdot and MitoView signals exhibited the five cancer cell lines, as evidenced by the perfect overlap observed in the merged image on the right column of Figure 3.49. The findings confirm that cationic Pdots show potential in several methods for visualizing nuclei and allocating organelles.

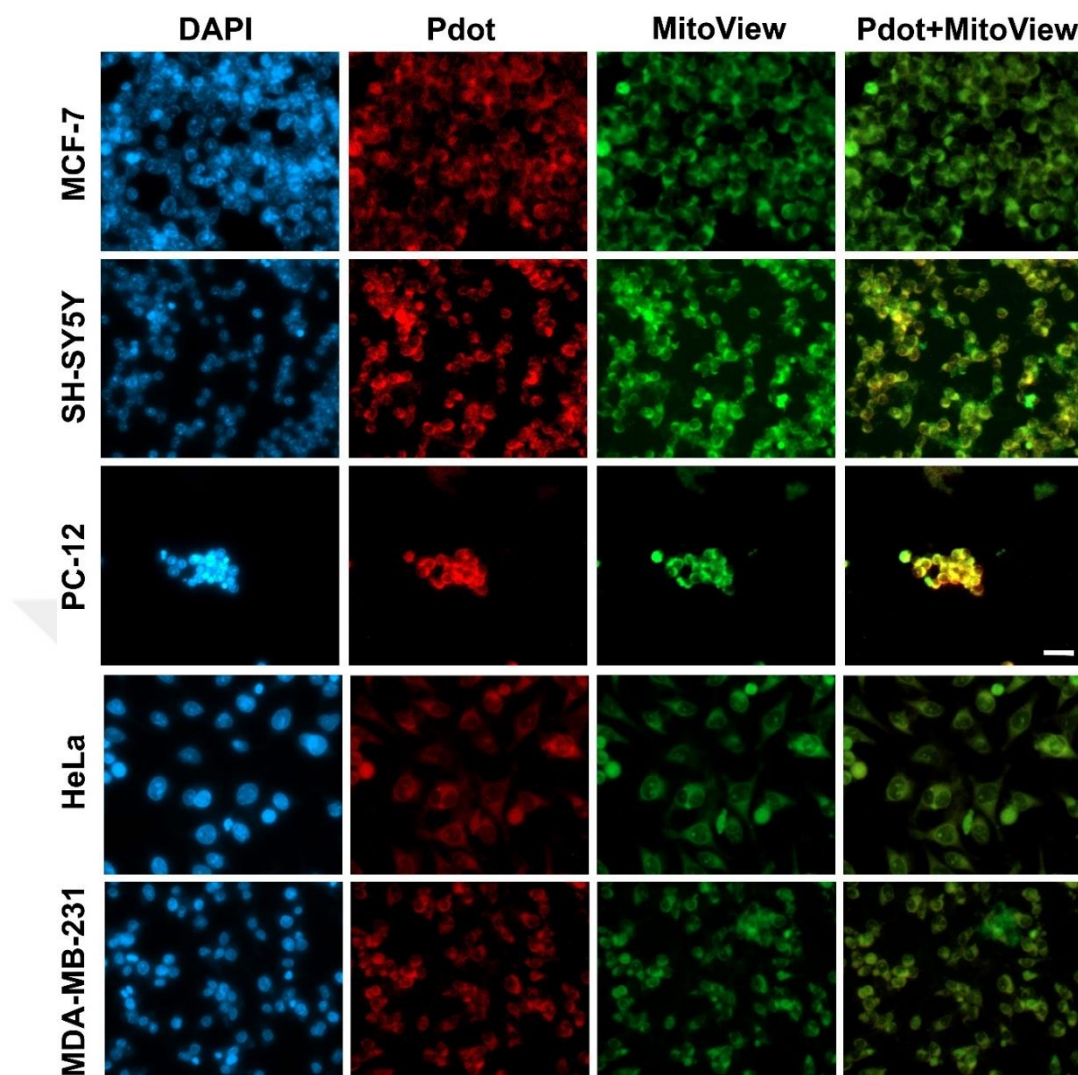


Figure 3.49. Fluorescence microscopy images of MCF-7, SH-SY5Y, PC-12, HeLa, and MDA-MB-23 cells, cultured with Pdot and labeled with DAPI and MitoView (blue: DAPI, red: Pdot, green: MitoView, scale bar: 20 μ m).

The graph illustrates the impact of applying NIR emissive Pdot on the viability of MCF-7, SH-SY5Y, PC-12, HeLa, and MDA-MB-231 cells, as well as the control groups depicted in Figure. The viability of the cells treated with NIR emissive Pdot was obtained as, %83, %79, %80, %70, and %70 viability for MCF-7, SH-SY5Y, PC-12, HeLa, and MDA-MB-231 cells, respectively (Figure 3.50). According to our prior research, Pdot can accumulate in either the nucleus or cytoplasm, depending on the specific type of cell. It has been demonstrated that Pdot can potentially interact with DNA, as reported by Ozenler et al. in 2019. The interaction between Pdots and DNA might lead to a decrease in cell proliferation, which is a crucial point to consider when

evaluating cell viability. Cell viability at the end of the 48th hour was compared between groups treated with NIR emissive Pdot, resulting in a partial decrease in cell viability.

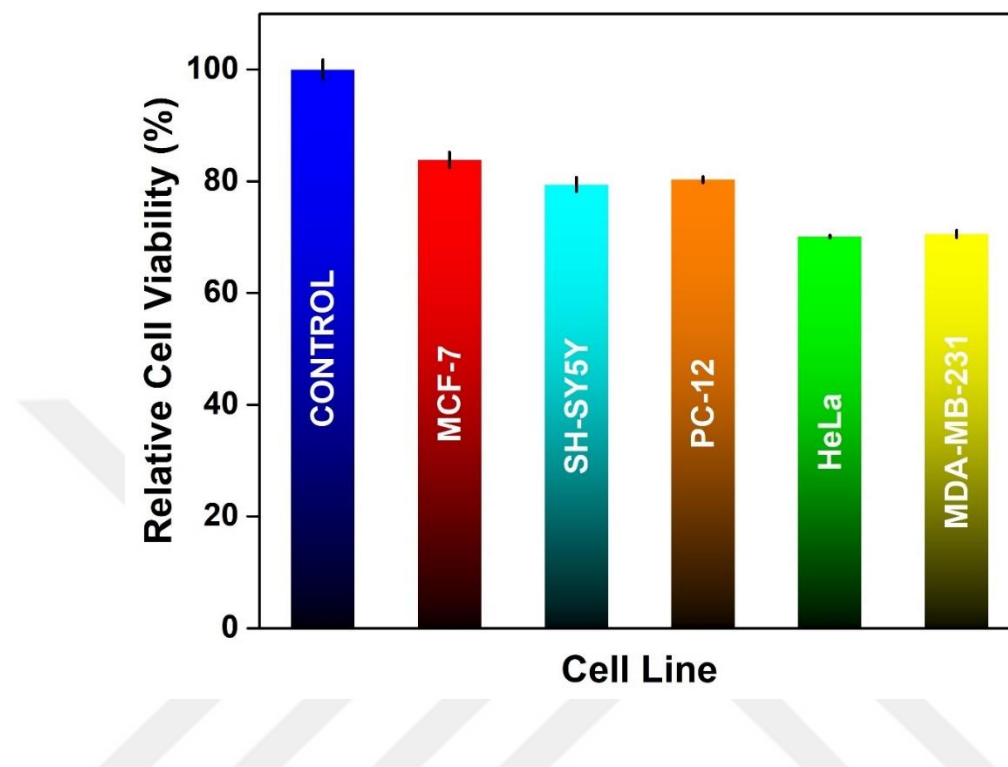


Figure 3.50. Relative Cell viability results obtained from Alamar blue analysis of Pdot cytotoxicity on MCF-7, SH-SY5Y, PC-12, HeLa, and MDA-MB-231 cells.

3.5. Optimization and Characterization of imaging parameters with Pdots in standard 3D cell culture models

The utilization of the hanging drop approach has been proposed to produce 3D tumoroid models. Extensive investigations on the development of 3D tumoroid models have been performed in this subject. MCF-7, SH-SY5Y, PC-12, HeLa, and MDA-MB-231 cells were used to produce 3D tumoroid models with a spheroid structure using the hanging drop technique. For each cell line, 10 μL droplets containing 25×10^3 and 50×10^3 cells were dropped into the central region of the petri dish. Subsequently, 2 mL of PBS solution was introduced into the petri dishes to preserve the moisture level of the surroundings, and the petri dish was carefully inverted and closed. Microscopic images were taken at 0, 4, and 24 hours of incubation using a light microscope. MCF-7 cells

were first observed within the initial 4 hours period, and a densely packed arrangement was observed under light microscopy after 24 hours (Figure 3.51).

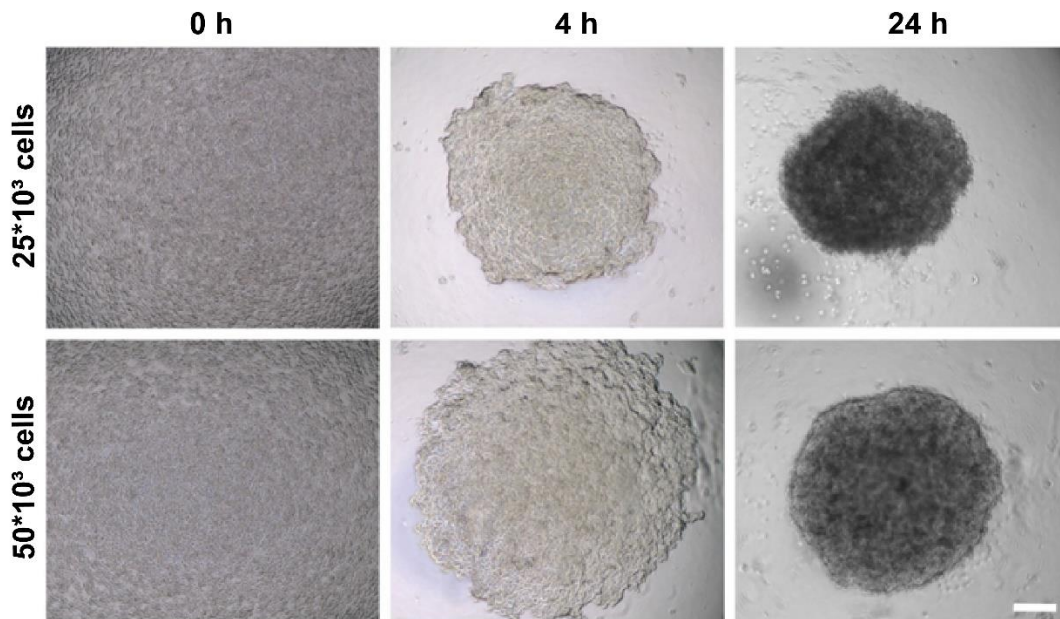


Figure 3.51. Light microscope analysis images of MCF-7 spheroids using 25×10^3 and 50×10^3 cell numbers produced by the hanging drop method. (Scale bar: 200 μm).

When SH-SY5Y cell lines were utilized, the formation of spheroid structures was shown to be more spread out during the initial 4-hour incubation phase. However, these spheroids achieved compact after 24 hours (Figure 3.52). Different cell lines exhibit varying behaviors when producing 3D models, which is a well-established and typical phenomenon directly associated with the cell's membrane receptors.

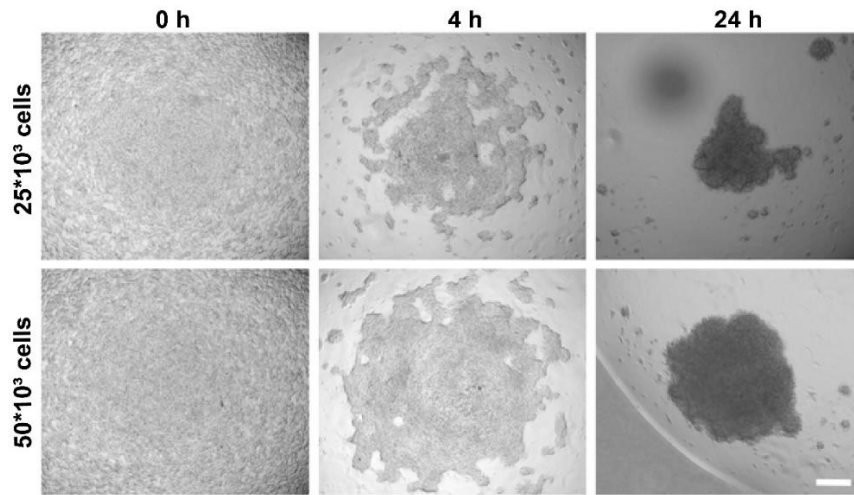


Figure 3.52. Light microscope analysis images of SH-SY5Y spheroids using 25×10^3 and 50×10^3 cell numbers produced by the hanging drop method. (Scale bar: 200 μm).

When PC-12 cell lines were used, it was observed that the production of spheroid formations was more dispersed during the first 4-hour incubation phase. Nevertheless, these spheroids attained a compact structure after 24 hours (Figure 3.53). Various cell lines have diverse characteristics in the production of 3D models, a phenomenon that is widely recognized and commonly linked to the cell's membrane receptors.

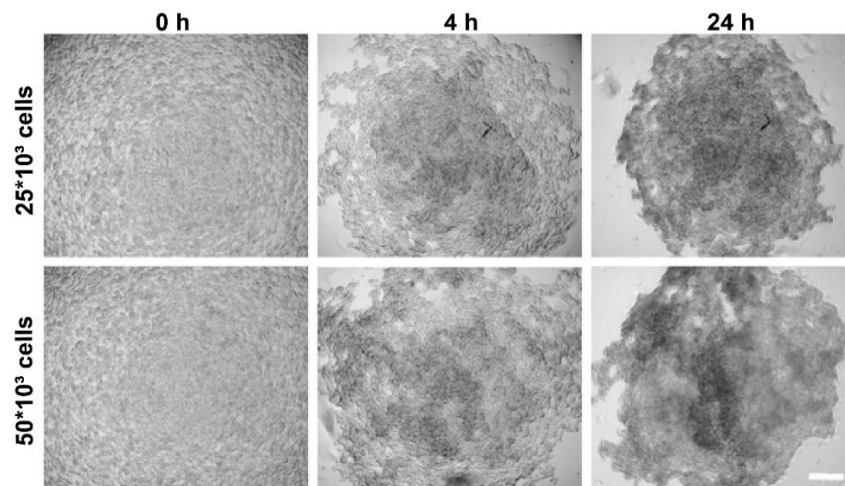


Figure 3.53. Light microscope analysis images of PC-12 spheroids using 25×10^3 and 50×10^3 cell numbers produced by the hanging drop method. (Scale bar: 200 μm).

As shown in Figure 3.54, spheroid structures with 25×10^3 and 50×10^3 cell numbers could not be observed in HeLa cells using the hanging drop method. Then, after 24 hours of incubation with a cell count of 10×10^3 , the spheroid structure was obtained (Figure 3.55). The formation of 3D spheroid structures was observed for both 25×10^3 and 50×10^3 cell numbers in all other cell lines except HeLa cells.

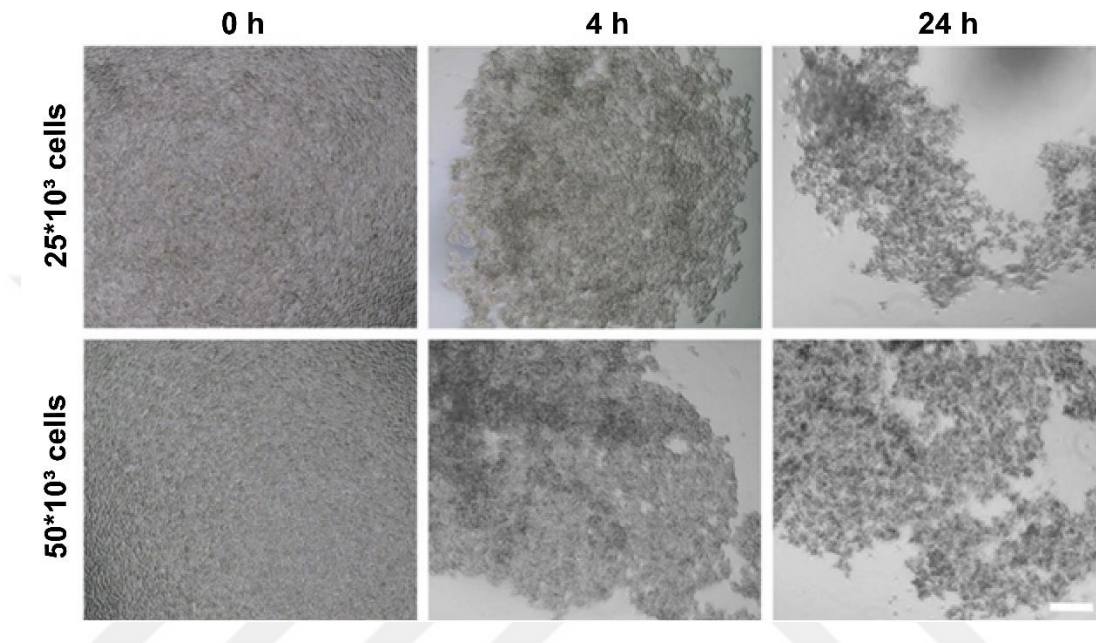


Figure 3.54. Light microscopy analysis images of HeLa spheroids using a cell count of 25×10^3 and 50×10^3 produced by the hanging drop method. (Scale bar: 200 μm).

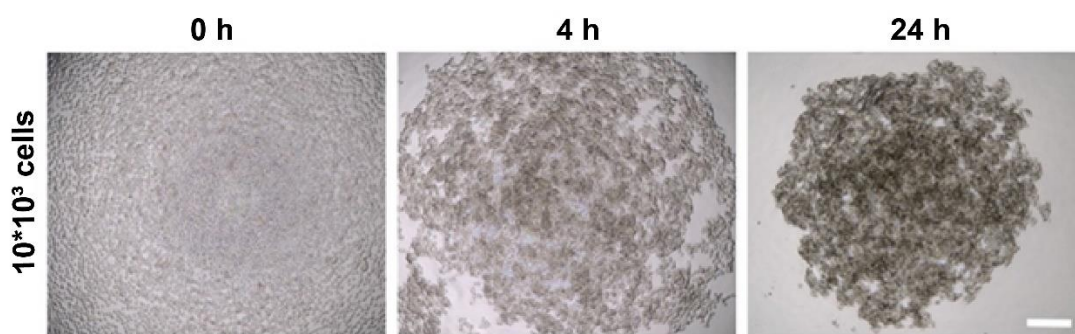


Figure 3.55. Light microscopy analysis images of HeLa spheroids using a cell count of 10×10^3 produced by the hanging drop method. (Scale bar: 200 μm).

The formation of a compact structure in MDA-MB-231 cells was observed during the first 4 hours, and by the end of 24 hours, it was apparent under a light microscope (Figure 3.56).

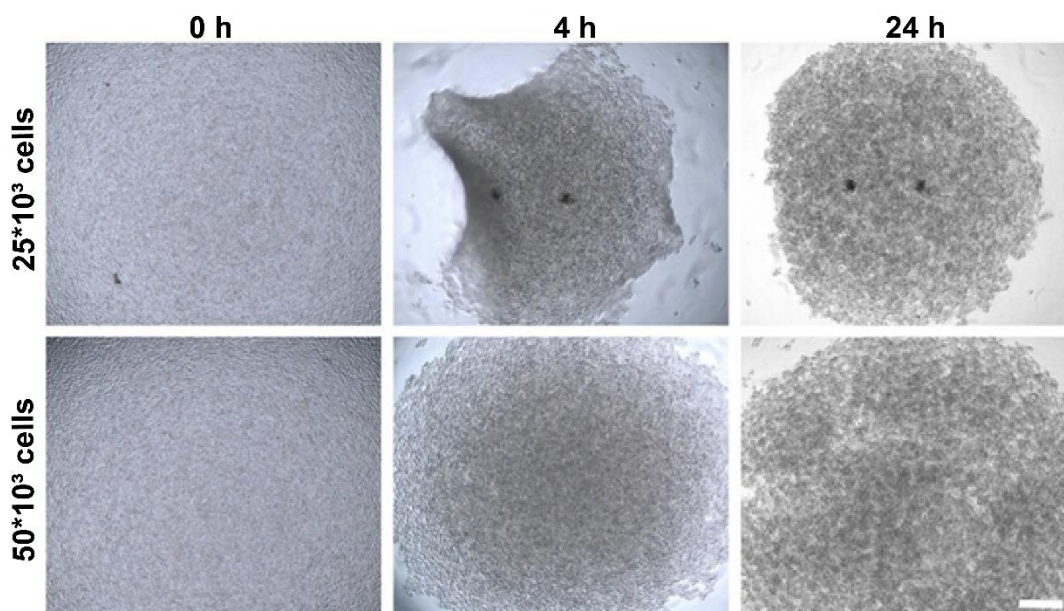


Figure 3.56. Light microscope analysis images of MDA-MB-231 spheroids using 25×10^3 and 50×10^3 cell numbers produced by the hanging drop method. (Scale bar: 200 μm).

The Pdot application was performed utilizing the obtained 3D tumor spheroids, and fluorescence imaging was carried out using Calcein Green and DAPI after 24 hours of incubation (Figure 3.57). Subsequently, the behavior of Pdots in three-dimensional tumor models was examined. After performing a cell viability study, the hanging drop approach was used to produce 3D tumoroid models. To do this, 10 μL droplets containing 25×10^3 cells of MDA-MB-231, MCF-7, and Pc-12 cells, 50×10^3 cells of SH-SY5Y cells, and 1×10^4 cells of HeLa cells were placed into the central region of the petri dish. Subsequently, 2 mL of PBS solution was introduced into the petri dishes to preserve the moisture level of the surroundings, and the petri dish was carefully turned and closed. After 24 hours of incubation, spheroid formations were formed for each cell line. Afterward, the spheroids were transferred to 96 microplates and performed Pdot application, imaging, and characterization procedures. The spheroids were cultured in a media treated with Pdot for a duration of 24 hours. Once the incubation period ended,

the medium was removed and the cell cytoplasm (green) and cell nucleus (blue) were labeled with Pdot through Calcein Green and DAPI staining, respectively. Calcein Green staining not only enables visualization of the cytoplasm, but also indicates the presence of living cells. This allows us to determine whether there is a decrease in cell viability following the application of Pdot in the produced spheroid forms. Subsequently, spheroids were fixed using a 4% solution of paraformaldehyde and examined using a fluorescence microscope.

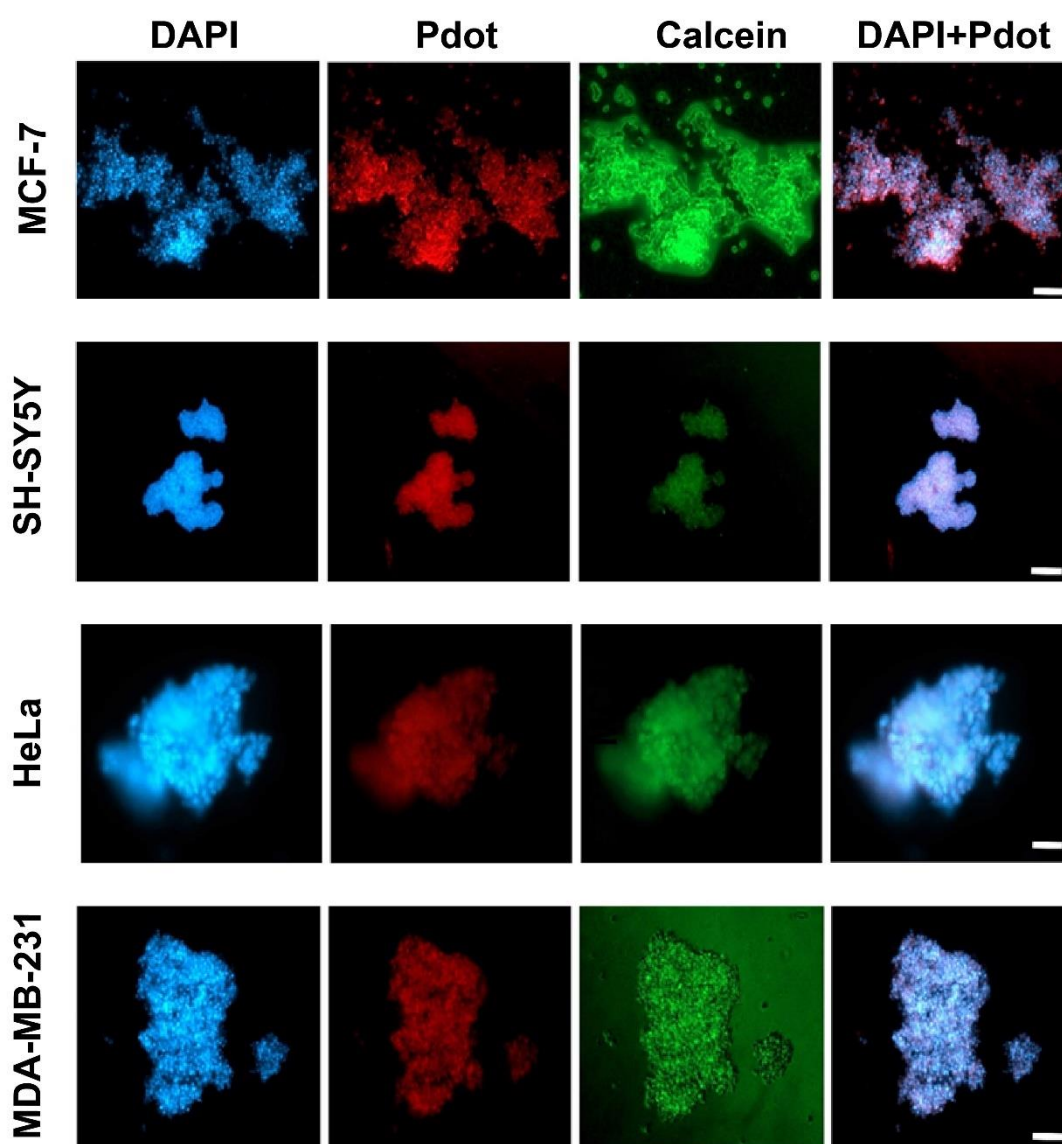


Figure 3.57. Fluorescence microscope image of Pdot-treated MCF-7, SH-SY5Y, HeLa and MDA-MB-231 spheroid in 96 microplates (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 100 μ m).

The Figure 3.57 displays the results of fluorescence microscopy examination on HeLa, MDA-MB-231, MCF-7, and SH-SY5Y spheroids treated with Pdots. The analysis included DAPI, Pdot, and Calcein Green staining. Within this part, the spheroids obtained through the hanging drop technique were initially moved to 96 microplates. Subsequently, DAPI, Pdot, and Calcein Green stainings were carried out in the same 96 microplates. The cells were cultured in Pdot for 24 hours, followed by staining with DAPI and Calcein Green. However, the examination of the microscope images showed that the cells were unable to keep their desired spherical form and characteristics in the 96 microplates, thereby failing to satisfy the requirements. Because of the imaging problem, it is challenging to provide a precise assessment of Pdot penetration when DAPI and Pdot images are superimposed. The spheroids formed were unable to maintain their three-dimensional spheroid structure after the application of Pdot, DAPI, and Calcein Green in 96-well microplates. The subsequent examination of roundness also verified this. Roundness is an essential feature for the formation of 3D spheroid and tumoroid formations. When the value approaches 1, it signifies that the spheroid possesses an optimal structure.⁸⁵ Roundness is defined as follows in equation 3.1:

$$\text{Roundness} = 4\pi (\text{area} / \text{perimeter}^2) \quad (3.1)$$

The roundness analysis results of HeLa, MDA-MB-231, MCF-7, and SH-SY5Y spheroids, which were examined using fluorescence microscopy in 96 microplates, are displayed in Figure 3.58. The investigation determined the roundness of the spheroids in 96 microplates to be 0.58, 0.56, 0.41, and 0.34 for HeLa, MDA-MB-231, MCF-7, and SH-SY5Y cells, respectively. These results indicate that the shapes of the resulting spheroids differ considerably from the ideal. As previously stated, the cells were unable to preserve their spheroid structure on 96 microplates.

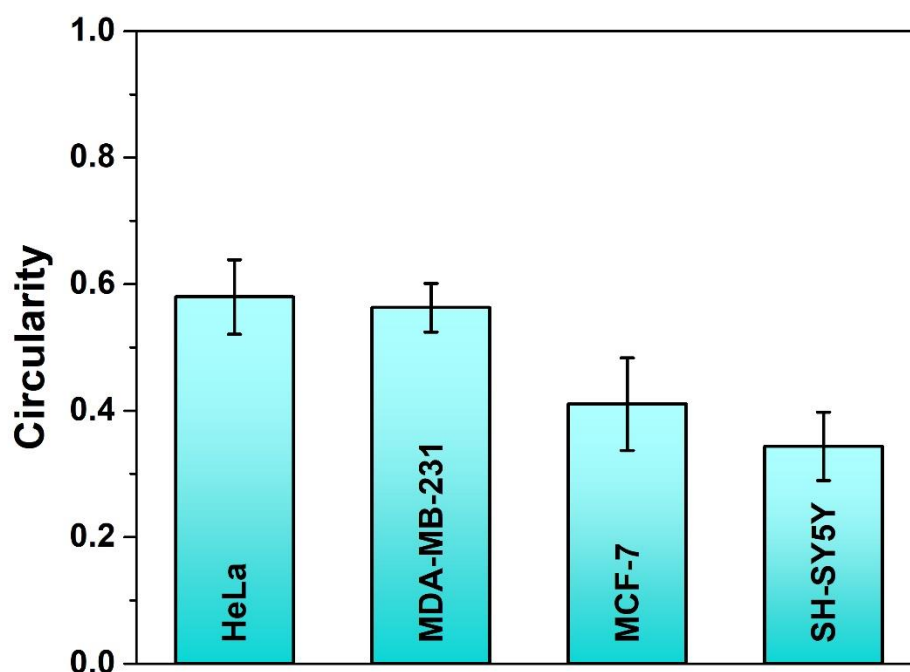


Figure 3.58. Roundness analysis of HeLa, MDA-MB-231, MCF-7 and SH-SY5Y spheroids performed by fluorescence microscopy analysis in 96 microplates.

After the analysis was performed using a fluorescent microscope on 96 microplates containing HeLa, MDA-MB-231, MCF-7, and SH-SY5Y cells, an alteration was carried out in the procedure. Instead of using 96 microplates, a glass surface was used to examine the spheroids produced by the hanging drop method. In this procedure, spheroids produced using the hanging drop technique were first placed in a 24-well micro-plate and then transferred onto UV-sterilized glass surfaces approximately 12 mm in diameter. Next, the spheroids were placed in a culture medium containing Pdot and allowed to incubate for 24 hours. Following the completion of incubation, the medium was removed, and the cell cytoplasm (green) and cell nucleus (blue) were labeled with Pdot through Calcein Green and DAPI staining, respectively. Next, the spheroids were fixed on the glass surface using a 4% paraformaldehyde solution. Once all of the steps were completed on the glass surface, the glass surface was removed from the microplate. Subsequently, the imaging step began, during which the penetration behavior of Pdot was examined using a fluorescence microscope. The application of Pdot, DAPI, and Calcein Green on the glass surface enabled the preservation of the spheroids' three-dimensional structure through the impact of surface tension. This approach also allowed for better visualization in fluorescence microscopy

analysis as compared to using 96 microplates. Furthermore, the roundness examined on these spheroids also confirms this finding. The roundness of the spheroids was determined to be 0.76, 0.86, 0.87, 0.77, and 0.83 for HeLa, MDA-MB-231, MCF-7, SH-SY5Y, and PC-12 cells, respectively, as shown in Figure 3.59. The results indicated that the spheroids obtained were nearly ideal.

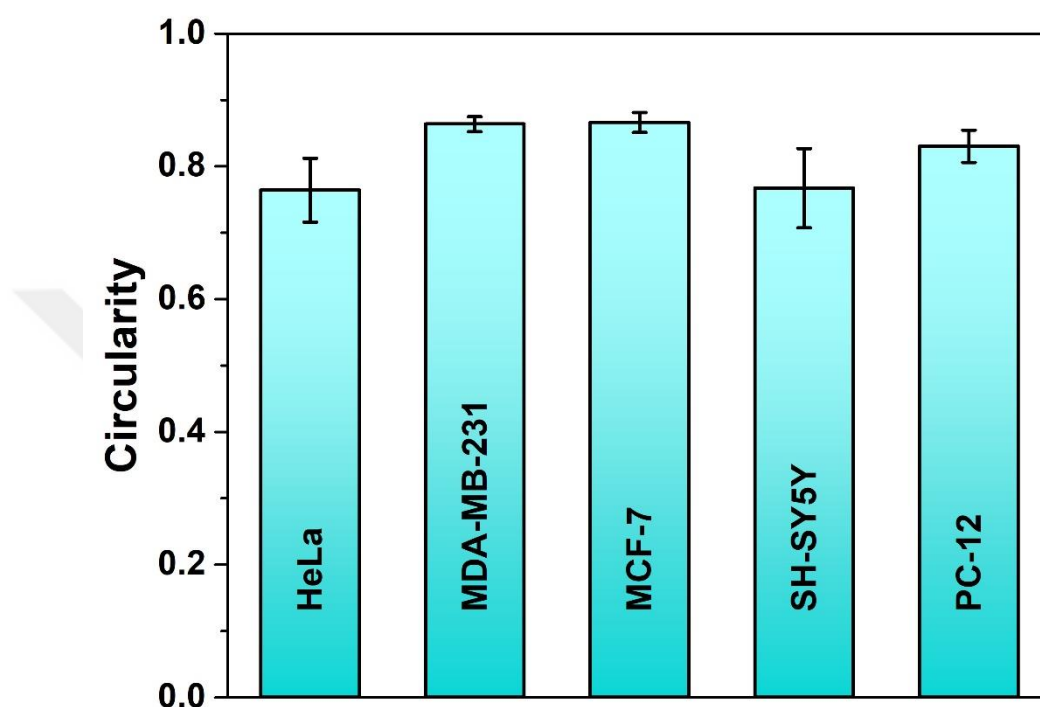


Figure 3.59. Roundness analysis of HeLa, MDA-MB-231, MCF-7, SH-SY5Y and PC-12 spheroids performed by fluorescence microscopy analysis on a glass surface.

Then, the spheroids produced with MCF-7 cells were placed in a mixture containing Pdot for 24 hours. Subsequently, the cell nucleus and cytoplasm were labeled using DAPI and Calcein Green dyes. Following that, the location and penetration of Pdot were thoroughly examined using fluorescence microscopy. Figure 3.60A displays the results of the fluorescence microscopy examination performed on MCF-7 spheroids treated with NIR emissive Pdot. The analysis focused on the presence of DAPI, Calcein Green, and Pdot. Figure 3.60B illustrates the penetration behavior of Pdot and DAPI from the outer wall to the center of the MCF-7 spheroid. In this examination, DAPI exhibited a notable localization into the outer layer of the spheroid, although its intensity decreased in the central region of the spheroid. The density graphs depicted in

Figure 3.60B were derived from measurements taken over the diameter of the spheroid at specific intervals (100, 200, 300, 400, 500, 600, 800, and 1000 μm). DAPI density exhibited an obvious decrease at distances of 300, 400, 500, and 600 μm from the center of the spheroid. However, an increase in DAPI density was detected at distances of 800 and 1000 μm when approaching the exterior wall of the spheroid. DAPI was observed to penetrate well into the outer wall of the spheroid, however, its intensity decreased in the inner regions. Similarly, the fluorescence intensity measurement for Pdot demonstrates a distinct pattern: as we approach the core of the spheroid, the intensity of Pdot increases at distances of 100, 200, 300, and 400 μm . This contrasts with DAPI, which does not exhibit the same trend. Furthermore, NIR emissive Pdot can penetrate deeper into the spheroid, as depicted in Figure 3.60B.

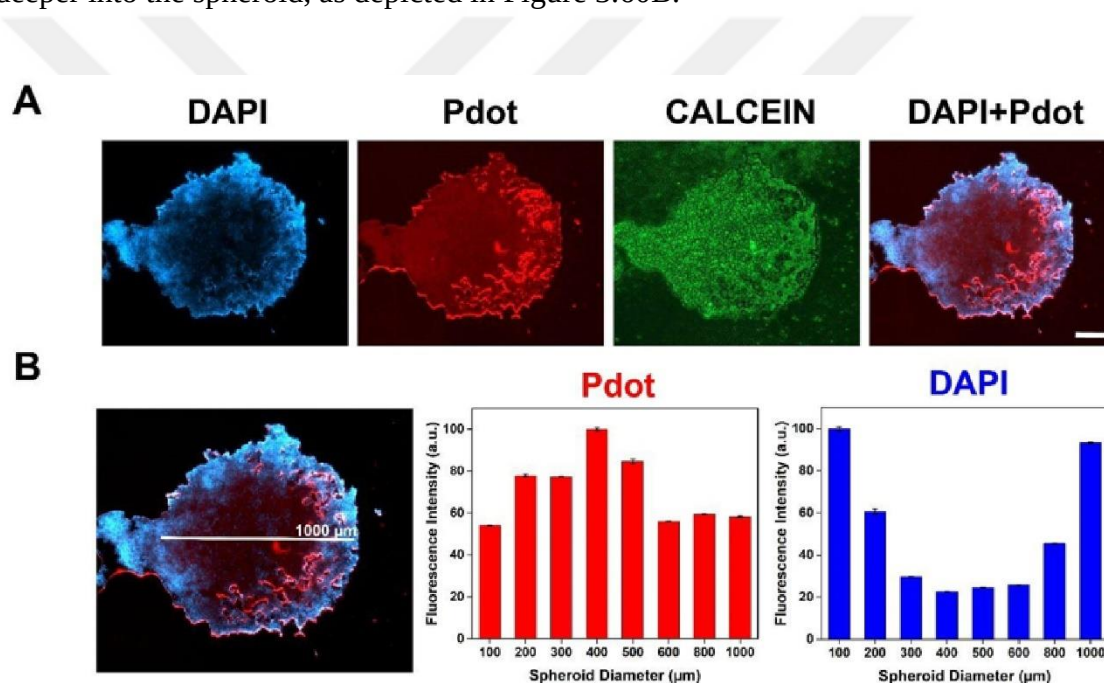


Figure 3.60. A) Fluorescence microscope image of 3D MCF-7 spheroid with Pdot applied on glass surface (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 200 μm). B) Pdot and DAPI penetration behavior along the diameter of the Pdot-treated MCF-7 spheroid.

Figure 3.61A shows the fluorescent microscope images of the SH-SY5Y spheroid treated with Pdot after having DAPI, Calcein Green, and Pdot staining. Figure 3.61B illustrates the patterns of Pdot and DAPI penetration across the diameter of the SH-SY5Y spheroid. A fluorescence microscopy examination was carried out to observe the nucleus, Pdot, and cell cytoplasm. The images obtained showed high cell viability.

By superimposing these images, the localization of Pdot and its penetration capabilities were compared to that of DAPI. Upon close examination of the overlay image of DAPI and Pdot, it becomes evident that DAPI is concentrated more densely at the outside periphery of the spheroid, but gradually decreases in intensity towards the center. The density graphs shown in Figure 3.61B similarly depict the penetration behavior observed in the photographs. The density of DAPI was quantified at specific distances of 50, 200, 400, 600, 800, 1000, 1200, and 1400 μm along the diameter of the spheroid. It was obvious that the density decreased gradually towards the core of the spheroid. There was an apparent decrease in the density of DAPI from 50 μm to 600 μm , while the density of Pdot increased (Figure 3.61B). However, in contrast to DAPI, it has been noted that Pdot is specifically localized in the central region of the spheroid, which is inaccessible to DAPI. The density graphs presented in Figure 3.61B demonstrate that NIR emissive Pdot is primarily localized in the central regions of the spheroid (200, 400, and 600 μm), while DAPI staining is less intense.

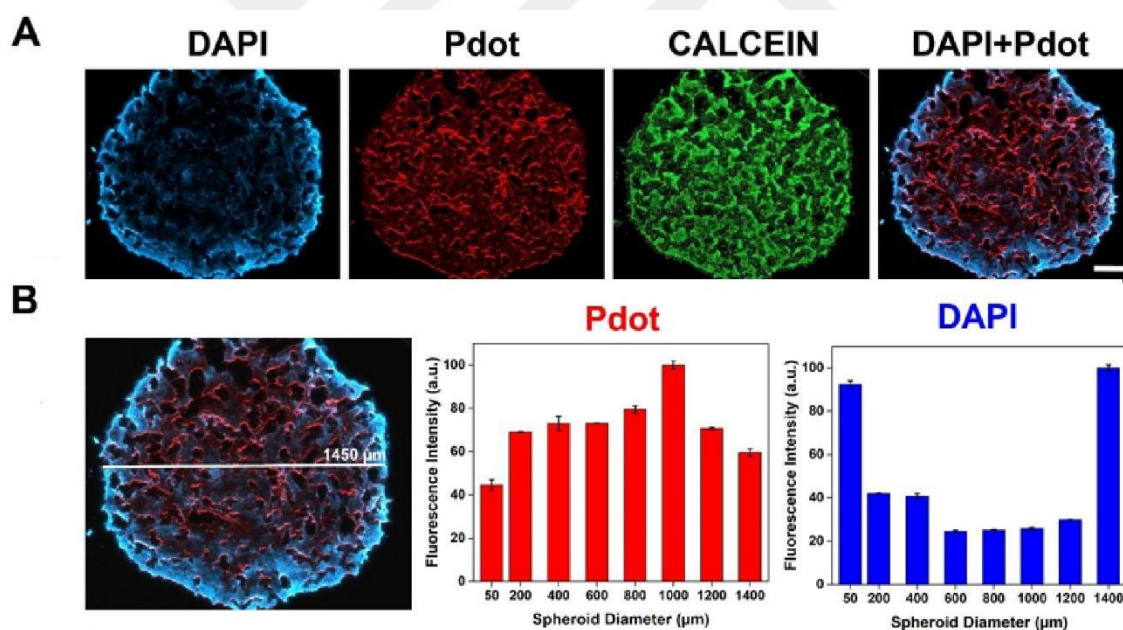


Figure 3.61. A) Fluorescence microscope image of 3D SHSY-5Y spheroid with Pdot applied on glass surface (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 200 μm). B) Pdot and DAPI penetration behavior along the diameter of the Pdot-treated SHSY-5Y spheroid.

Figure 3.62A shows the results of fluorescence microscopy analysis carried out on a spheroid derived from PC-12 cells. The spheroid was produced using the hanging

drop method and then incubated in a medium containing NIR emissive Pdot for 24 hours. The characterization of the spheroid was subsequently performed. Fluorescence microscopy analysis was performed individually for DAPI, Pdot, and Calcein Green. Like other spheroids, the penetration of Pdot was stronger in the central portion of the spheroids derived from PC-12 cells, where the intensity of DAPI staining was low. Figure 3.62B demonstrates the density analysis graphs performed for Pdot and DAPI. The measurements were taken at intervals of 100 μm along the diameter of the spheroid, specifically at 100, 200, 300, 400, 500, 600, 700, and 800 μm . Upon evaluation of the produced graphs, it was observed that the density of DAPI decreased from the outside wall to the interior section of the spheroid at distances of 200, 300, 400, and 500 μm . In contrast, the density of NIR emissive Pdot increased, indicating a clear demonstration of Pdot's ability to penetrate inside the spheroid.

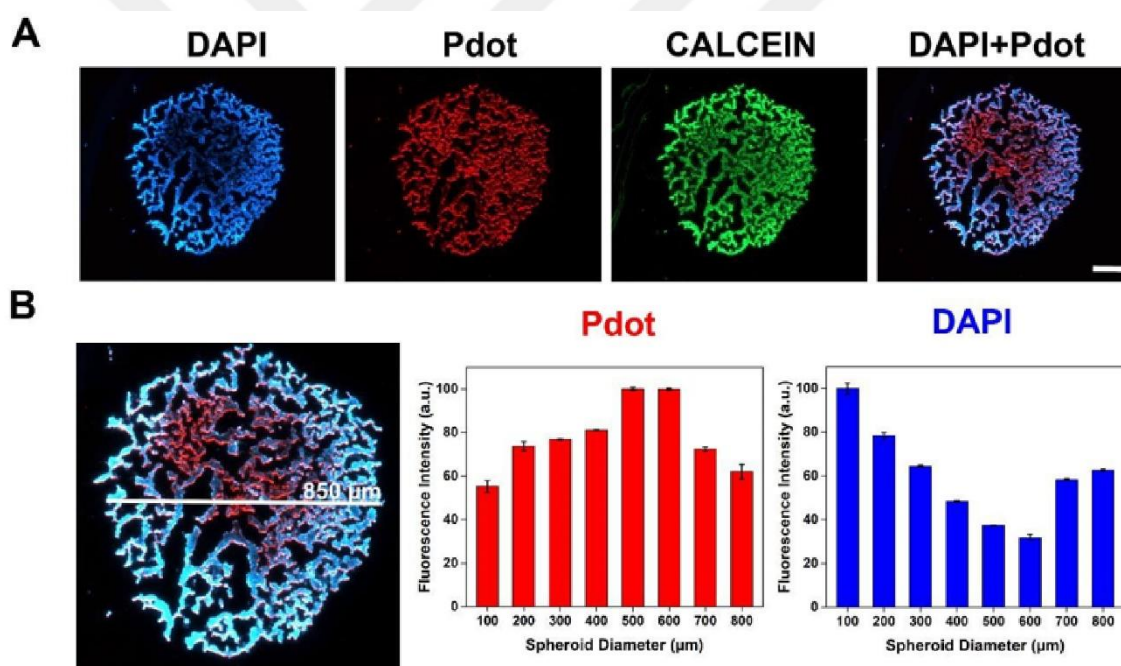


Figure 3.62. A) Fluorescence microscope image of 3D PC-12 spheroid with Pdot applied on glass surface (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 200 μm). B) Pdot and DAPI penetration behavior along the diameter of the Pdot-treated PC-12 spheroid.

Furthermore, the MDA-MB-231 cancer cell line was used to produce 3D spheroid structures by the hanging drop technique. Subsequently, the penetration of Pdot was examined in the resulting spheroid structures after 24 hours of incubation.

Figure 3.63A displays the findings of fluorescent microscopy examination of MDA-MB-231 spheroid, which underwent Pdot characterization. As shown in the figure, the final structure exhibits a significant degree of permeability in comparison to other spheroids and did not retain its structural integrity according to expectations during the characterization steps. Structurally weak spheroids may lose their compact structure during analysis processes such as Pdot application, Calcein Green and DAPI staining, and fixation treatments that are applied following the hanging drop method. The lack of rigidity in the spheroid obtained during the initial phase leads to its disintegration in subsequent steps, occasionally appearing multiple smaller spheroids. Figure 3.63B displays the density measurements of Pdot and DAPI following fluorescence microscopy examination of spheroids derived from the MDA-MB-231 cell line. The measurements were taken at intervals of 25 μm along the diameter of the spheroid, specifically at 50, 75, 100, 125, 150, 175, and 180 μm . Due to the spheroid's inability to maintain its structure during the characterization process and the absence of diffusion restriction caused by its size, the penetration of NIR emissive Pdot could not be observed, unlike in SH-SY5Y, MCF-7, and PC-12 spheroids. However, the density of DAPI seems to diminish in the central regions of the spheroid, similar to what is observed in other cells. The findings obtained can be attributed to the distinct surface molecules of various cell lines. Each cell line forms spheroids with diverse structures, dependent upon the surface adhesion molecules it possesses. One cell line can produce extremely compact spheroids, while another cell line can produce less dense structures under the same conditions. According to literature studies, many adhesion molecules can facilitate the production of spheroids in breast cancer cells. Although E-cadherin is involved in the process of spheroid formation in MCF-7 cells, MDA-MB-231 cells lack cadherin molecules. In these cells, spheroid formation is instead supported by the relationship between collagen I and integrin $\beta 1$.

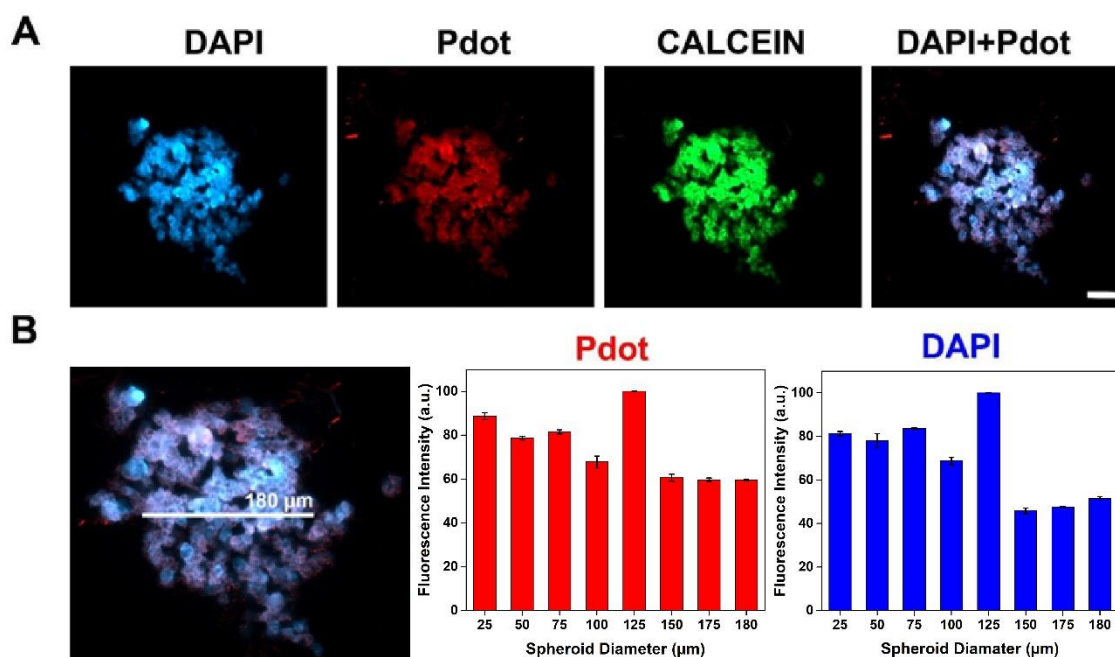


Figure 3.63. A) Fluorescence microscope image of 3D MDA-MB-231 spheroid with Pdot applied on glass surface (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 200 μm). B) Pdot and DAPI penetration behavior along the diameter of the Pdot-treated MDA-MB-231 spheroid.

Figure 3.64A shows the results of the fluorescence microscopy examination conducted on the HeLa spheroid produced by the hanging drop method, following the application of Pdot. As previously mentioned, various cancer cells form 3-D structures with a variety of densities. After 24 hours of employing the hanging drop approach, the HeLa cells exhibited a very disorganized structure. In addition, the application of Pdot, followed by Calcein Green and DAPI staining, results in an interruption of the 3D structure, causing it to lose its spherical shape and structural integrity. Figure 3.64B displays the intensity measurements performed for NIR emissive Pdot and DAPI following fluorescence microscopy examination. The measurements were taken at intervals of 50 μm along the diameter of the spheroid, specifically at 50, 100, 150, 200, 300, 400, 500, and 550 μm . Based on density measurements, there was no apparent increase in Pdot penetration behavior from the spheroid wall into the inner part. Similarly, DAPI does not exhibit any distinct behavior. The lack of diffusion restriction in the loosely formed spheroid prevented clear observation of Pdot penetration, unlike in other spheroid models.

For this investigation, an overall of five different cancer cell lines were employed to produce three-dimensional tumor models based on spheroids. Each cell line exhibits distinct activity within 3D structures. While both PC-12 cells and the SH-SY5Y cell line are neural tissue cells, the hanging drop approach resulted in PC-12 cells forming a less dense 3D structure, whereas the SH-SY5Y cell line generated a very compact 3D spheroid shape. Cell adhesion molecules play a crucial function in facilitating cell-cell contact and tissue formation. The behavior of SH-SY5Y cells is linked to cell adhesion molecules (CAM) and extracellular matrix (ECM) proteins. The elevated levels of CAM protein and N-cadherin expression in SH-SY5Y spheroids are linked to the compact structure of the spheroid. Furthermore, the MDA-MB-231 cells yielded more loosely formed spheroids in comparison to the MCF-7 cells. It is linked to the adhesion molecule integrin $\beta 1$ and claudin4 of MDA-MB-231 cells. According to literature studies, many adhesion molecules play a significant role in the creation of spheroids by breast cancer cells. E-cadherin contributes to the creation of spheroids in MCF-7 cells; however cadherin molecules are not present in MDA-MB-231 cells. In these cells, spheroid formation can be assisted by the connection between collagen I and integrin $\beta 1$. The synthesis of N-cadherin and catenin, which are responsible for the creation of cell-cell contacts, is well-established in the process of spheroid formation in HeLa cells. Cadherins form connections with the actin cellular structure, leading to powerful adhesion between cells.

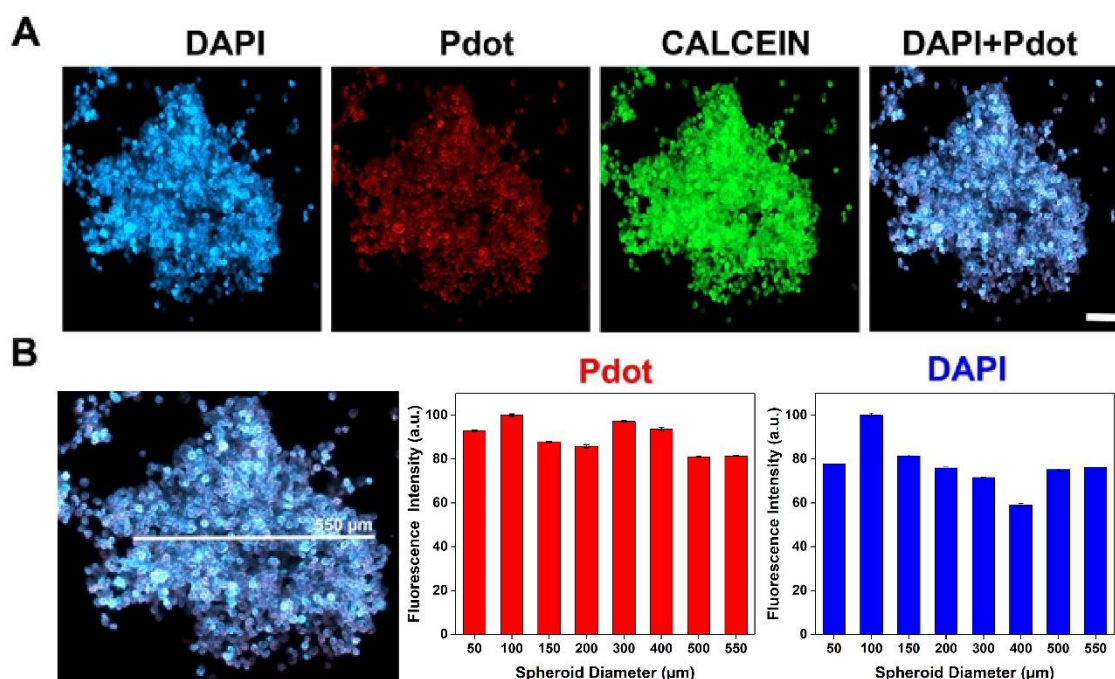


Figure 3.64. A) Fluorescence microscope image of 3D HeLa spheroid with Pdot applied on glass surface (blue: DAPI, red: Pdot, green: calcein green) (Scale bar: 200 μm). B) Pdot and DAPI penetration behavior along the diameter of the Pdot-treated HeLa spheroid.

Following the study with NIR emissive Pdot, 3D spheroids were visualized using MitoView Green, a fluorescent dye known for its ability to specifically label mitochondria, as shown in Figure 3.65. The study was performed with MCF-7, SH-SY5Y, and PC-12 tumoroid spheroids which successfully form a spheroid. The investigations revealed interesting patterns of Pdot and MitoView Green being present in specific areas of the spheroids, suggesting that Pdot may be entering the mitochondria. This behavior corresponds with previous research indicating that specific nanoparticle compositions, such as quantum dots and carbon dots, can pass through cellular membranes and accumulate in subcellular organelles, including mitochondria. This is due to their size and surface characteristics.^{86–88} Nevertheless, it is crucial to recognize that the occurrence of Pdot staining in areas that do not completely match with MitoView Green staining suggests interactions with components of cells other than mitochondria. These interactions may include membrane binding, distribution within the cytoplasm, or connection with other cellular components. Further investigation is necessary to comprehend the molecular mechanisms that are responsible for these

interactions. This research may offer valuable insights into the pathways through which Pdots are taken up by cells and transported inside them.

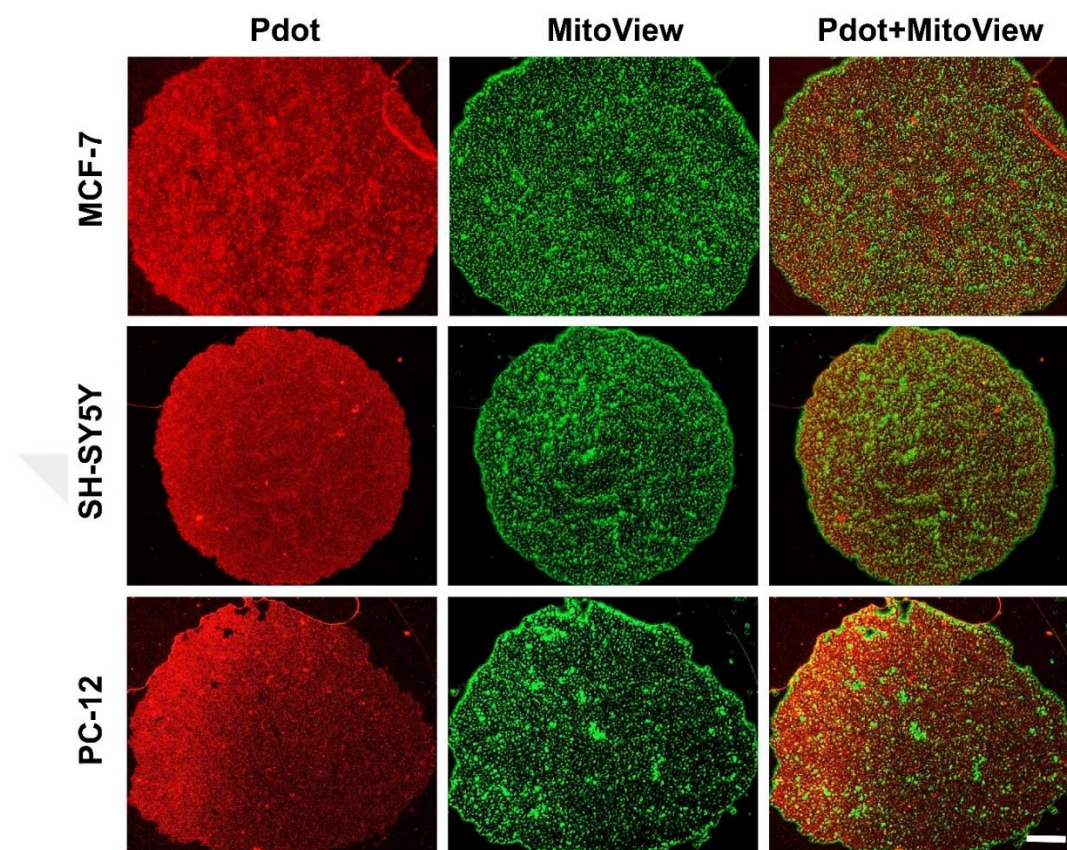


Figure 3.65. Fluorescence microscopy images of 3D MCF-7, SH-SY5Y, and PC-12 cell spheroids cultured with Pdot and labeled with MitoView Green (red: Pdot, green: MitoView, scale bar: 200 μ m).

The obtained images were analyzed via ImageJ program to examine the localization of NIR emissive Pdot in the tumoroid spheroids as compared to MitoView. As seen in Figure 3.66, Figure 3.67, and Figure 3.68, the penetration behavior of the NIR emissive Pdot and MitoView were investigated through the yellow line in the spheroid images. According to the analyses, it can be said that NIR emissive Pdot has an emission in all parts of the spheroid but MitoView labels the mitochondria. The findings show that the NIR emissive Pdot has better penetration capability than MitoView in the MCF-7, SH-SY5Y, and PC-12 cell spheroids.

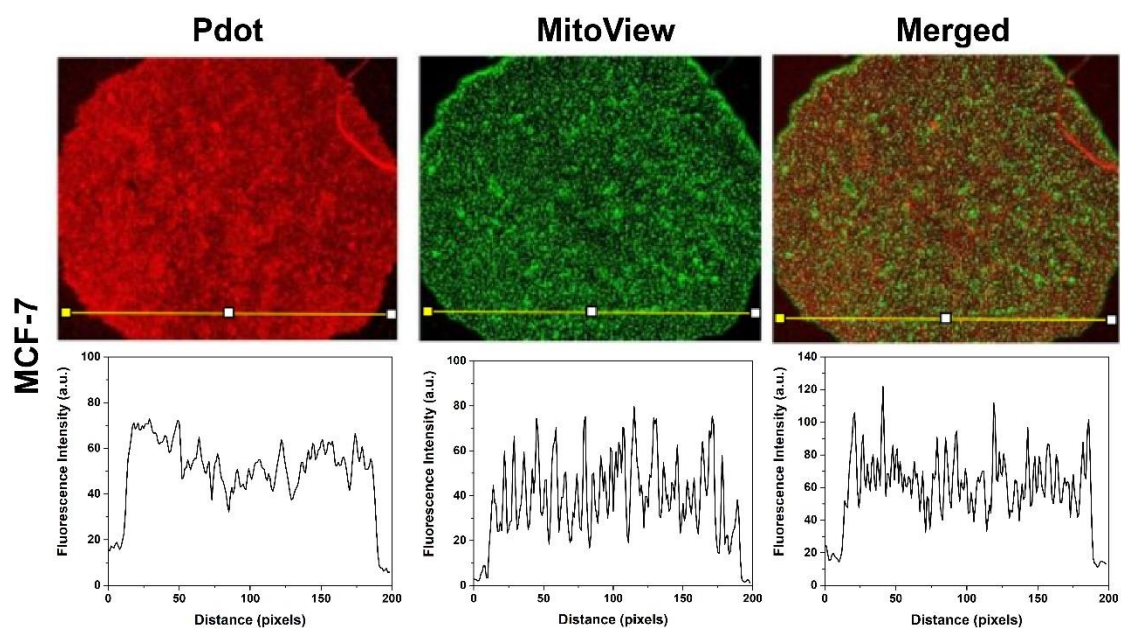


Figure 3.66. Fluorescence intensity profile of Pdot and MitoView for MCF-7 cells.

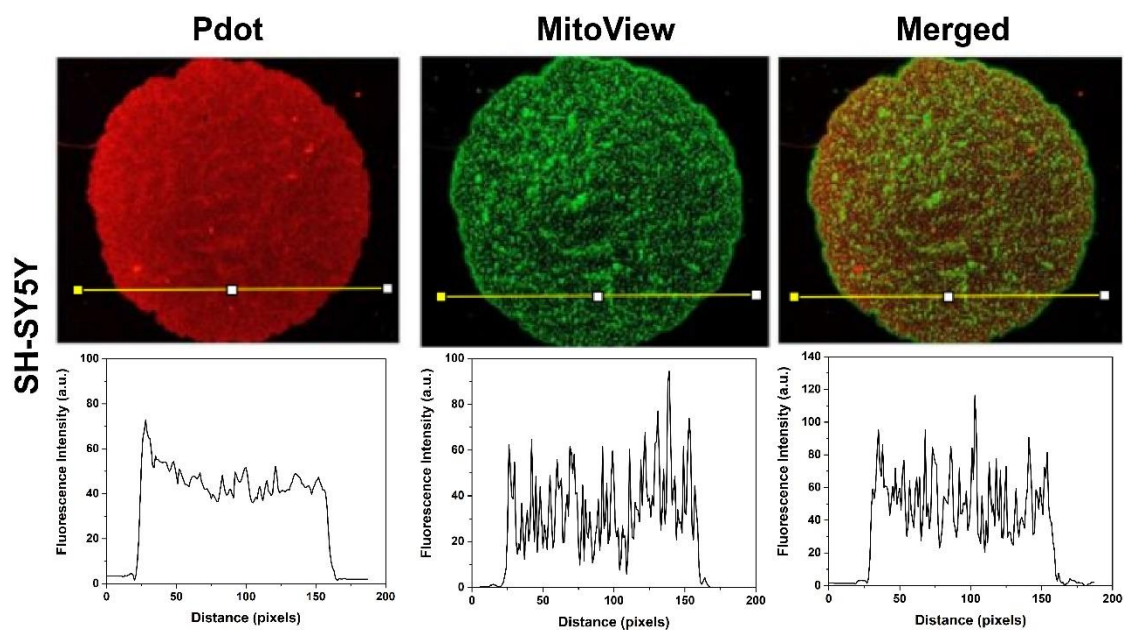


Figure 3.67. Fluorescence intensity profile of Pdot and MitoView for SH-SY5Y cells.

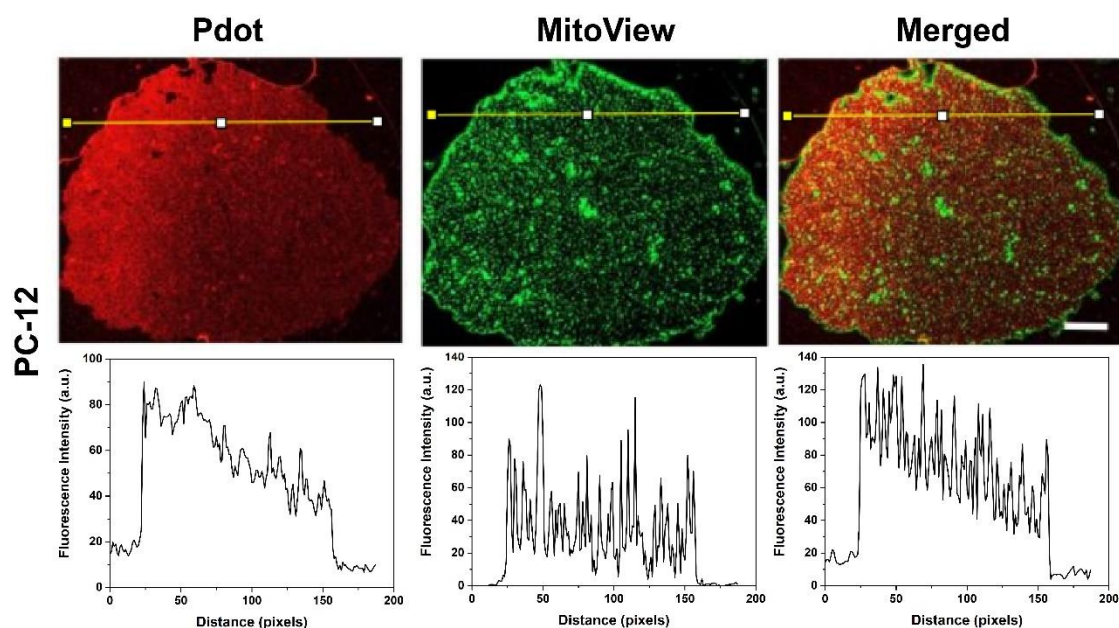


Figure 3.68. Fluorescence intensity profile of Pdot and MitoView for PC-12 cells.

Also, the distribution and colocalization of Mitoview and Pdots were evaluated using the Karhunen-Loeve transform (KLT) and principal components analysis (PCA) with the assistance of FIJI software. This analysis can be seen in Figure 3.69. In this study, the red channel (Pdots) and green channel (MitoView) of the overlay images were processed using a specified number of pixels. The examination of the KLT/PCA results indicate variations in the distribution of PC-12, SH-SY5Y, and MCF-7 tumor spheroids. This research can offer useful insights into the features of these cell lines and their interactions with Pdots and MitoView staining. The closeness of the data points to the red axis in the KLT/PCA color space indicates that the changes in the dataset correspond better with the direction indicated by the red channel (Pdots). This positioning suggests a greater concentration or visibility of characteristics detected by the red channel, indicating a stronger relationship or interaction between the Pdots and the PC-12 cell-based spheroids. The red-stained features in the PC-12 spheroids could indicate expected cellular structures or components, such as cytoplasmic regions or membrane-associated proteins, that display unique fluorescence properties when interacting with the Pdots. The aggregation of data points along the red axis may indicate consistent patterns or distributions of these characteristics across the PC-12 spheroids, signifying a more homogeneous response to the Pdots treatment. On the other hand, the collection of data points that are closer to the starting location in the

KLT/PCA color space for SH-SY5Y and MCF-7 cell-based spheroids suggests a more spread-out distribution with less noticeable alignment in a certain direction. The distribution of the dataset indicates that the variations in the data are not substantially correlated with any specific color channel. This means that all color channels (red, green, and potentially blue) contribute equally to the dataset. The clustering of data points around the origin may indicate a more diverse response to the Pdots and MitoView stains within the SH-SY5Y and MCF-7 spheroids. This diversity is reflected in a broader spectrum of fluorescence intensities and patterns observed in various locations of the spheroids. The SH-SY5Y and MCF-7 spheroids may have different cell forms, rates of proliferation, or metabolic functions compared to PC-12 spheroids. This might lead to a wider and more scattered distribution of fluorescence signals in the KLT/PCA color space.

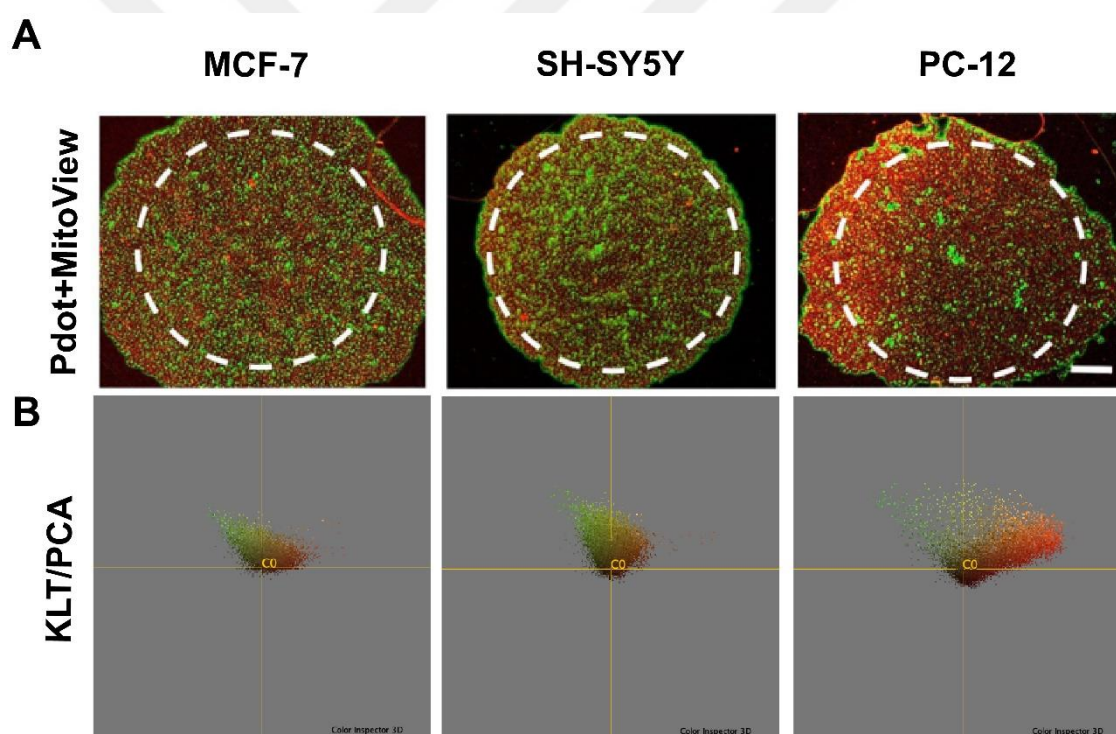


Figure 3.69. Pdot distribution and colocalization of Mitoview analysis with KLT/PCA for MCF-7, SH-SY5Y, and PC-12 cell spheroids.

Overall, the differences in the distribution of data points in the KLT/PCA color space between PC-12 and SH-SY5Y MCF-7 cell-based tumor spheroids indicate unique relationships between the cells and the Pdots and Mito View. These changes may also

suggest disparities in cellular characteristics and behaviors. It is important to mention that the stability of cationic Pdots and their high surface charge ($> +40$ mV) play a critical role in determining how they are taken up by cells and where they are located within the cells. The presence of a positive surface charge enables electrostatic interactions with negatively charged cell membranes, hence enhancing the process of cellular internalization through endocytosis pathways. Moreover, the surface charge of nanoparticles is crucial in defining their intracellular destiny, as it affects their interactions with intracellular components and subcellular organelles.^{89–93} In summary, our research adds to the increasing amount of evidence that supports the diverse uses of cationic Pdots in medical imaging. It also emphasizes their promise for understanding complex cellular processes in both normal and diseased conditions. Additional investigation into the molecular basis for Pdot-cell interactions and the optimization of their physicochemical characteristics would unquestionably improve their effectiveness and adaptability in biomedical research and therapeutic applications.

CHAPTER 4

CONCLUSION

In this thesis, the synthesis of non-ionic donor-acceptor based conjugated polymers for obtaining cationized Pdots that can maintain their colloidal stability has been studied. The NIR-emissive conjugated Pdots provide significant advantages for imaging tumor spheroids with their fluorescence characteristics in the NIR region. The most significant finding underlined by this thesis is that NIR emissive Pdots provide high-resolution imaging within the tumor without losing signal intensity. So, the penetration behavior of Pdots was examined within the tumoroid models. To obtain NIR emissive polymers, three different polymerization types were used. These are oxidative polymerization, direct arylation polymerization, and stille polymerization, respectively. With these reactions, a total of 10 NIR emissive polymers were tried to be obtained and they were named from P1 to P10.

The first three NIR emissive polymers were synthesized by oxidative polymerization. These were named P1, P2, and P3, respectively. Structurally, these polymers are based on a monomer with a D-A-D structure. Each polymer consists of different monomers, and the most significant difference is the donor units they have. For example, P1 has BuMT as a donor, P2 has DiBuT as a donor, and P3 has EDOT as a donor. What they have in common is the use of the same acceptor unit, BBT. As a result, three different NIR emissive polymers were synthesized through oxidative polymerization based on D-A-D. After being structurally characterized, its optical properties were examined. The absorbance analysis revealed the maximum wavelengths for P1, P2, and P3 to be 660 nm, 630 nm, and 940 nm, respectively. The subsequent emission analysis revealed that the maximum wavelengths for P1 and P2 polymers were 860 nm and 856 nm, respectively. However, emission analysis could not be performed for P3, because it was not possible to access an instrument that could perform emission analysis by stimulating its absorbance at its maximum wavelength. Considering all these, it is understood that P1, P2, and P3, synthesized through oxidative polymerization, have strong emissions in the NIR region. When comparing the results based on the absorbance maximum wavelengths, P3 stands out for its polymerization

ability. Also, P1 has a greater maximum absorbance wavelength than the P2. The donor units' aliphatic groups are the main reason for this correlation. For example, it was understood that P3 is the polymer with the highest number of monomers, with the advantage of the ethylene dioxy groups it has, because the red shift increases as the number of monomers increases in polymers formed from D-A-D monomers. The presence of a butoxy group in only the 3rd position of the donor unit in P1 provides a relative advantage in polymerization compared to P2, resulting in a longer polymer chain.

Subsequently, two different NIR emissive polymers, P4 and P5, were tried to be synthesized using direct arylation polymerization. Before synthesizing P4 and P5, the DArP protocol was tested using BuMT. The test confirms the successful implementation of the DArP protocols. However, D-A based polymerization was not carried out. This can be interpreted as the long aliphatic groups that the donor groups have, as well as the BBT acceptor unit not being suitable for this DArP.

The synthesis and characterization of NIR emissive polymers continued with P6-P10 by stille polymerization. The optical properties of the resulting five different stille polymerization products were characterized. A comparison between these products shows that as the molar ratios of bilaterally stannylated EDOT in stille polymerization protocols increase, the maximum wavelength tends to be higher than other wavelengths. The absorption λ_{max} was characterized as 755 nm, 741 nm, 705 nm, 680 nm, and 630 nm for P6-10 polymers, respectively. This trend was attributed to the butoxy group of DiBuT because the aliphatic group reduced the polymerization efficiency. In contrast to DiBuT, EDOT has no steric hindrance. Therefore, as the EDOT content of NIR emissive polymers increases, the absorption λ_{max} values also increase. Then, the trend in the emission spectra of Stille polymerization products (P6–10) was investigated. It shows that the maximum wavelength tends to be higher as the molar ratios of bilaterally stannylated EDOT in the stille polymerization protocols go up. The emission λ_{max} was characterized as 881 nm, 883 nm, 875 nm, 810 nm, and 775 nm for P6-10 polymers, respectively. This trend was attributed to the same reason as mentioned above. Therefore, as the EDOT content of NIR emissive polymers increases, the emission λ_{max} values also increase.

After the synthesis and characterization of NIR emissive polymers were completed, Pdot was obtained from P1 using the miniemulsion technique to be used in

bioapplications as a P1 model polymer. Ultrasonic emulsification forms single-chain Pdots by combining a nonionic D-A-D type polymer, P1, with an amphiphilic compound, CTAB. The process produces Pdots that have a significantly positive surface charge of $+56.5 \text{ mV} \pm 9.5$ and an average hydrodynamic radius of 12 nm. Optical analysis demonstrates that these Pdots emit NIR light with a peak wavelength of 860 nm. In environments where diffusion is limited, such as tissues, Pdots with a positive charge demonstrate a notable benefit. This is achieved by employing various tumor spheroid models, including MCF-7, SH-SY5Y, PC-12, MDA-MB-231, and HeLa.

Analysis of tumor spheroids from MCF-7, SH-SY5Y, PC-12, MDA-MB-231, and HeLa cell lines using fluorescence microscopy shows that the intensity profile of Pdots confirms their extensive penetration into the center regions of the tumoroid models. Various parameters, including size, surface charge, surface chemistry, and mechanical qualities, influence the penetration of tumor spheroids by Pdots. Soft cationic Pdots possess distinct physicochemical properties that can offer benefits to Pdots, especially those with flexible and soft structures. Furthermore, cells may absorb these Pdots more effectively than other types of nanoparticles. Significantly, the use of a mitochondria staining dye demonstrates a convergence between the regions stained by Pdots and the dye in three tumor spheroid models (MCF-7, SH-SY5Y, and PC-12). The findings indicate that Pdots have a significant ability to penetrate dense media and tumors, with a mean free path of roughly 1 μm . The study shows that when PC-12 and SH-SY5Y MCF-7 tumor spheroids are looked at in the KLT/PCA color space, the cells and the Pdots and Mito View interact. Additionally, there may be differences in cellular characteristics and behavior. It's important to note that cationic Pdots must be stable in a colloidal state and have a high surface charge of more than +40 mV in order for cells to take them up and find their way inside the cells.

When compared to nanoparticles with a negative charge, Pdots are much better at penetrating tumor spheroids because they are soft and have a positive charge. Pdots have the potential to improve the effectiveness of cancer treatment by utilizing their distinct physicochemical features to enhance the transport and distribution of therapeutic drugs into solid tumors. Gaining a deeper understanding of the mechanisms that allow Pdots to enter tumor spheroids can help develop more effective nanomedicine platforms for treating cancer.

REFERENCES

1. Chiotaki, R.; Polioudaki, H.; Theodoropoulos, P. A. Differential Nuclear Shape Dynamics of Invasive Andnon-Invasive Breast Cancer Cells Are Associated with Actin Cytoskeleton Organization and Stability. *Biochemistry and Cell Biology* **2014**, 92 (4), 287–295. <https://doi.org/10.1139/bcb-2013-0120>.
2. Bishop, C. J.; Kozielski, K. L.; Green, J. J. Exploring the Role of Polymer Structure on Intracellular Nucleic Acid Delivery via Polymeric Nanoparticles. *Journal of Controlled Release* **2015**, 219, 488–499. <https://doi.org/10.1016/j.jconrel.2015.09.046>.
3. Weissleder, R.; Pittet, M. J. Imaging in the Era of Molecular Oncology. *Nature* **2008**, 452 (7187), 580–589. <https://doi.org/10.1038/nature06917>.
4. Weissleder, R. Scaling down Imaging: Molecular Mapping of Cancer in Mice. *Nat Rev Cancer* **2002**, 2 (1), 11–18. <https://doi.org/10.1038/nrc701>.
5. Antaris, A. L.; Chen, H.; Diao, S.; Ma, Z.; Zhang, Z.; Zhu, S.; Wang, J.; Lozano, A. X.; Fan, Q.; Chew, L.; Zhu, M.; Cheng, K.; Hong, X.; Dai, H.; Cheng, Z. A High Quantum Yield Molecule-Protein Complex Fluorophore for near-Infrared II Imaging. *Nat Commun* **2017**, 8 (1), 15269. <https://doi.org/10.1038/ncomms15269>.
6. Yang, Q.; Ma, Z.; Wang, H.; Zhou, B.; Zhu, S.; Zhong, Y.; Wang, J.; Wan, H.; Antaris, A.; Ma, R.; Zhang, X.; Yang, J.; Zhang, X.; Sun, H.; Liu, W.; Liang, Y.; Dai, H. Rational Design of Molecular Fluorophores for Biological Imaging in the NIR-II Window. *Advanced Materials* **2017**, 29 (12). <https://doi.org/10.1002/adma.201605497>.
7. Yang, Q.; Hu, Z.; Zhu, S.; Ma, R.; Ma, H.; Ma, Z.; Wan, H.; Zhu, T.; Jiang, Z.; Liu, W.; Jiao, L.; Sun, H.; Liang, Y.; Dai, H. Donor Engineering for NIR-II

- Molecular Fluorophores with Enhanced Fluorescent Performance. *J Am Chem Soc* **2018**, *140* (5), 1715–1724. <https://doi.org/10.1021/jacs.7b10334>.
8. Zandt, B.-J.; Liu, J. H.; Veruki, M. L.; Hartveit, E. All Amacrine Cells: Quantitative Reconstruction and Morphometric Analysis of Electrophysiologically Identified Cells in Live Rat Retinal Slices Imaged with Multi-Photon Excitation Microscopy. *Brain Struct Funct* **2017**, *222* (1), 151–182. <https://doi.org/10.1007/s00429-016-1206-0>.
 9. Bashkatov, A. N.; Genina, E. A.; Kochubey, V. I.; Tuchin, V. V. Optical Properties of Human Skin, Subcutaneous and Mucous Tissues in the Wavelength Range from 400 to 2000 Nm. *J Phys D Appl Phys* **2005**, *38* (15), 2543–2555. <https://doi.org/10.1088/0022-3727/38/15/004>.
 10. Hong, G.; Antaris, A. L.; Dai, H. Near-Infrared Fluorophores for Biomedical Imaging. *Nat Biomed Eng* **2017**, *1* (1), 0010. <https://doi.org/10.1038/s41551-016-0010>.
 11. Owens, E. A.; Henary, M.; El Fakhri, G.; Choi, H. S. Tissue-Specific Near-Infrared Fluorescence Imaging. *Acc Chem Res* **2016**, *49* (9), 1731–1740. <https://doi.org/10.1021/acs.accounts.6b00239>.
 12. Naczynski, D. J.; Tan, M. C.; Zevon, M.; Wall, B.; Kohl, J.; Kulesa, A.; Chen, S.; Roth, C. M.; Riman, R. E.; Moghe, P. V. Rare-Earth-Doped Biological Composites as in Vivo Shortwave Infrared Reporters. *Nat Commun* **2013**, *4* (1), 2199. <https://doi.org/10.1038/ncomms3199>.
 13. Li, D.; Jing, P.; Sun, L.; An, Y.; Shan, X.; Lu, X.; Zhou, D.; Han, D.; Shen, D.; Zhai, Y.; Qu, S.; Zbořil, R.; Rogach, A. L. Near-Infrared Excitation/Emission and Multiphoton-Induced Fluorescence of Carbon Dots. *Advanced Materials* **2018**, *30* (13). <https://doi.org/10.1002/adma.201705913>.
 14. Hong, G.; Diao, S.; Chang, J.; Antaris, A. L.; Chen, C.; Zhang, B.; Zhao, S.; Atochin, D. N.; Huang, P. L.; Andreasson, K. I.; Kuo, C. J.; Dai, H. Through-

- Skull Fluorescence Imaging of the Brain in a New near-Infrared Window. *Nat Photonics* **2014**, 8 (9), 723–730. <https://doi.org/10.1038/nphoton.2014.166>.
15. Welsher, K.; Liu, Z.; Sherlock, S. P.; Robinson, J. T.; Chen, Z.; Daranciang, D.; Dai, H. A Route to Brightly Fluorescent Carbon Nanotubes for Near-Infrared Imaging in Mice. *Nat Nanotechnol* **2009**, 4 (11), 773–780. <https://doi.org/10.1038/nnano.2009.294>.
 16. Pu, K.; Mei, J.; Jokerst, J. V.; Hong, G.; Antaris, A. L.; Chattopadhyay, N.; Shuhendler, A. J.; Kurosawa, T.; Zhou, Y.; Gambhir, S. S.; Bao, Z.; Rao, J. Diketopyrrolopyrrole-Based Semiconducting Polymer Nanoparticles for In Vivo Photoacoustic Imaging. *Advanced Materials* **2015**, 27 (35), 5184–5190. <https://doi.org/10.1002/adma.201502285>.
 17. Jeong, J.-E.; Uddin, M. A.; Ryu, H. S.; Kim, H.-C.; Kang, M.; Joung, J. F.; Park, S.; Shim, S.-H.; Woo, H. Y. Green-, Red-, and Near-Infrared-Emitting Polymer Dot Probes for Simultaneous Multicolor Cell Imaging with a Single Excitation Wavelength. *Chemistry of Materials* **2020**, 32 (15), 6685–6696. <https://doi.org/10.1021/acs.chemmater.0c02172>.
 18. Ding, D.; Pu, K.-Y.; Li, K.; Liu, B. Conjugated Oligoelectrolyte-Polyhedral Oligomeric Silsesquioxane Loaded PH-Responsive Nanoparticles for Targeted Fluorescence Imaging of Cancer Cell Nucleus. *Chemical Communications* **2011**, 47 (35), 9837. <https://doi.org/10.1039/c1cc13237g>.
 19. Yang, Y.; Chen, J.; Yang, Y.; Xie, Z.; Song, L.; Zhang, P.; Liu, C.; Liu, J. A 1064 Nm Excitable Semiconducting Polymer Nanoparticle for Photoacoustic Imaging of Gliomas. *Nanoscale* **2019**, 11 (16), 7754–7760. <https://doi.org/10.1039/C9NR00552H>.
 20. Wu, C.; Chiu, D. T. Highly Fluorescent Semiconducting Polymer Dots for Biology and Medicine. *Angewandte Chemie International Edition* **2013**, 52 (11), 3086–3109. <https://doi.org/10.1002/anie.201205133>.

21. Li, S.; Wang, X.; Hu, R.; Chen, H.; Li, M.; Wang, J.; Wang, Y.; Liu, L.; Lv, F.; Liang, X.-J.; Wang, S. Near-Infrared (NIR)-Absorbing Conjugated Polymer Dots as Highly Effective Photothermal Materials for *In Vivo* Cancer Therapy. *Chemistry of Materials* **2016**, *28* (23), 8669–8675. <https://doi.org/10.1021/acs.chemmater.6b03738>.
22. Wei, Q.; Xu, D.; Li, T.; He, X.; Wang, J.; Zhao, Y.; Chen, L. Recent Advances of NIR-II Emissive Semiconducting Polymer Dots for In Vivo Tumor Fluorescence Imaging and Theranostics. *Biosensors (Basel)* **2022**, *12* (12), 1126. <https://doi.org/10.3390/bios12121126>.
23. Yang, C.; Liu, H.; Zhang, Y.; Xu, Z.; Wang, X.; Cao, B.; Wang, M. Hydrophobic-Sheath Segregated Macromolecular Fluorophores: Colloidal Nanoparticles of Polycaprolactone-Grafted Conjugated Polymers with Bright Far-Red/Near-Infrared Emission for Biological Imaging. *Biomacromolecules* **2016**, *17* (5), 1673–1683. <https://doi.org/10.1021/acs.biomac.6b00092>.
24. Zhang, Y.; Yu, J.; Gallina, M. E.; Sun, W.; Rong, Y.; Chiu, D. T. Highly Luminescent, Fluorinated Semiconducting Polymer Dots for Cellular Imaging and Analysis. *Chemical Communications* **2013**, *49* (74), 8256. <https://doi.org/10.1039/c3cc44048f>.
25. Yang, C.; Liu, H.; Zhang, Y.; Xu, Z.; Wang, X.; Cao, B.; Wang, M. Hydrophobic-Sheath Segregated Macromolecular Fluorophores: Colloidal Nanoparticles of Polycaprolactone-Grafted Conjugated Polymers with Bright Far-Red/Near-Infrared Emission for Biological Imaging. *Biomacromolecules* **2016**, *17* (5), 1673–1683. <https://doi.org/10.1021/acs.biomac.6b00092>.
26. Yu, J.; Wu, C.; Zhang, X.; Ye, F.; Gallina, M. E.; Rong, Y.; Wu, I.; Sun, W.; Chan, Y.; Chiu, D. T. Stable Functionalization of Small Semiconducting Polymer Dots via Covalent Cross-Linking and Their Application for Specific Cellular Imaging. *Advanced Materials* **2012**, *24* (26), 3498–3504. <https://doi.org/10.1002/adma.201201245>.

27. Chowdhury, P.; Chan, Y.-H. Recent Advances in D–A–D Based Pdots with NIR-II Fluorescence for Deep-Tissue Imaging. *Mol Syst Des Eng* **2022**, *7* (7), 702–719. <https://doi.org/10.1039/D2ME00034B>.
28. Wang, F.; Chen, H.; Liu, Z.; Mi, F.; Fang, X.; Liu, J.; Wang, M.; Lo, P. K.; Li, Q. Conjugated Polymer Dots for Biocompatible SiRNA Delivery. *New Journal of Chemistry* **2019**, *43* (36), 14443–14449. <https://doi.org/10.1039/C9NJ03277K>.
29. Özenler, S.; Yucel, M.; Tüncel, Ö.; Kaya, H.; Özçelik, S.; Yildiz, U. H. Single Chain Cationic Polymer Dot as a Fluorescent Probe for Cell Imaging and Selective Determination of Hepatocellular Carcinoma Cells. *Anal Chem* **2019**, *91* (16), 10357–10360. <https://doi.org/10.1021/acs.analchem.9b02300>.
30. Yucel, M.; Onbas, R.; Arslan Yildiz, A.; Yildiz, U. H. The Soft Nanodots as Fluorescent Probes for Cell Imaging: Analysis of Cell and Spheroid Penetration Behavior of Single Chain Polymer Dots. *Macromol Biosci* **2024**. <https://doi.org/10.1002/mabi.202300402>.
31. Xu, P.; Meng, Q.; Sun, H.; Yin, Q.; Yu, H.; Zhang, Z.; Cao, M.; Zhang, Y.; Li, Y. Shrapnel Nanoparticles Loading Docetaxel Inhibit Metastasis and Growth of Breast Cancer. *Biomaterials* **2015**, *64*, 10–20. <https://doi.org/10.1016/j.biomaterials.2015.06.017>.
32. Cai, Y.; Liang, P.; Tang, Q.; Yang, X.; Si, W.; Huang, W.; Zhang, Q.; Dong, X. Diketopyrrolopyrrole–Triphenylamine Organic Nanoparticles as Multifunctional Reagents for Photoacoustic Imaging-Guided Photodynamic/Photothermal Synergistic Tumor Therapy. *ACS Nano* **2017**, *11* (1), 1054–1063. <https://doi.org/10.1021/acsnano.6b07927>.
33. Davis, M. E.; Chen, Z.; Shin, D. M. Nanoparticle Therapeutics: An Emerging Treatment Modality for Cancer. *Nat Rev Drug Discov* **2008**, *7* (9), 771–782. <https://doi.org/10.1038/nrd2614>.

34. Chen, Z.; Zhao, P.; Luo, Z.; Zheng, M.; Tian, H.; Gong, P.; Gao, G.; Pan, H.; Liu, L.; Ma, A.; Cui, H.; Ma, Y.; Cai, L. Cancer Cell Membrane–Biomimetic Nanoparticles for Homologous-Targeting Dual-Modal Imaging and Photothermal Therapy. *ACS Nano* **2016**, *10* (11), 10049–10057.
<https://doi.org/10.1021/acsnano.6b04695>.
35. Kim, J.-H.; Park, K.; Nam, H. Y.; Lee, S.; Kim, K.; Kwon, I. C. Polymers for Bioimaging. *Prog Polym Sci* **2007**, *32* (8–9), 1031–1053.
<https://doi.org/10.1016/j.progpolymsci.2007.05.016>.
36. Hilderbrand, S. A.; Weissleder, R. Near-Infrared Fluorescence: Application to in Vivo Molecular Imaging. *Curr Opin Chem Biol* **2010**, *14* (1), 71–79.
<https://doi.org/10.1016/j.cbpa.2009.09.029>.
37. Hayashi, K.; Nakamura, M.; Miki, H.; Ozaki, S.; Abe, M.; Matsumoto, T.; Ishimura, K. Near-Infrared Fluorescent Silica/Porphyrin Hybrid Nanorings for In Vivo Cancer Imaging. *Adv Funct Mater* **2012**, *22* (17), 3539–3546.
<https://doi.org/10.1002/adfm.201200219>.
38. Li, K.; Liu, B. Water-Soluble Conjugated Polymers as the Platform for Protein Sensors. *Polym. Chem.* **2010**, *1* (3), 252–259.
<https://doi.org/10.1039/B9PY00283A>.
39. Feng, F.; He, F.; An, L.; Wang, S.; Li, Y.; Zhu, D. Fluorescent Conjugated Polyelectrolytes for Biomacromolecule Detection. *Advanced Materials* **2008**, *20* (15), 2959–2964. <https://doi.org/10.1002/adma.200800624>.
40. Wu, C.; Bull, B.; Szymanski, C.; Christensen, K.; McNeill, J. Multicolor Conjugated Polymer Dots for Biological Fluorescence Imaging. *ACS Nano* **2008**, *2* (11), 2415–2423. <https://doi.org/10.1021/nn800590n>.
41. Li, K.; Pan, J.; Feng, S.; Wu, A. W.; Pu, K.; Liu, Y.; Liu, B. Generic Strategy of Preparing Fluorescent Conjugated-Polymer-Loaded Poly(<sc>DL</Sc>

- lactide- Co -Glycolide) Nanoparticles for Targeted Cell Imaging. *Adv Funct Mater* **2009**, 19 (22), 3535–3542. <https://doi.org/10.1002/adfm.200901098>.
42. Tuncel, D.; Demir, H. V. Conjugated Polymer Nanoparticles. *Nanoscale* **2010**, 2 (4), 484. <https://doi.org/10.1039/b9nr00374f>.
 43. Becker, A.; Hessenius, C.; Licha, K.; Ebert, B.; Sukowski, U.; Semmler, W.; Wiedenmann, B.; Grötzinger, C. Receptor-Targeted Optical Imaging of Tumors with near-Infrared Fluorescent Ligands. *Nat Biotechnol* **2001**, 19 (4), 327–331. <https://doi.org/10.1038/86707>.
 44. Shu, X.; Royant, A.; Lin, M. Z.; Aguilera, T. A.; Lev-Ram, V.; Steinbach, P. A.; Tsien, R. Y. Mammalian Expression of Infrared Fluorescent Proteins Engineered from a Bacterial Phytochrome. *Science (1979)* **2009**, 324 (5928), 804–807. <https://doi.org/10.1126/science.1168683>.
 45. Cai, W.; Shin, D.-W.; Chen, K.; Gheysens, O.; Cao, Q.; Wang, S. X.; Gambhir, S. S.; Chen, X. Peptide-Labeled Near-Infrared Quantum Dots for Imaging Tumor Vasculature in Living Subjects. *Nano Lett* **2006**, 6 (4), 669–676. <https://doi.org/10.1021/nl052405t>.
 46. Grimsdale, A. C.; Leok Chan, K.; Martin, R. E.; Jokisz, P. G.; Holmes, A. B. Synthesis of Light-Emitting Conjugated Polymers for Applications in Electroluminescent Devices. *Chem Rev* **2009**, 109 (3), 897–1091. <https://doi.org/10.1021/cr000013v>.
 47. Li, K.; Liu, B. Polymer Encapsulated Conjugated Polymernanoparticles for Fluorescence Bioimaging. *J. Mater. Chem.* **2012**, 22 (4), 1257–1264. <https://doi.org/10.1039/C1JM14397B>.
 48. Nilsson, K. P. R.; Inganäs, O. Chip and Solution Detection of DNA Hybridization Using a Luminescent Zwitterionic Polythiophene Derivative. *Nat Mater* **2003**, 2 (6), 419–424. <https://doi.org/10.1038/nmat899>.

49. Kamat, S. V.; Puri, V.; Puri, R. K. Determination of the Optical Constants of a Vacuum Evaporated Polythiophene Thin Film. *Phys Scr* **2011**, *84* (4), 045801. <https://doi.org/10.1088/0031-8949/84/04/045801>.
50. Wakioka, M.; Nakamura, Y.; Wang, Q.; Ozawa, F. Direct Arylation of 2-Methylthiophene with Isolated $[\text{PdAr}(\mu\text{-O}_2\text{CR})(\text{PPh}_3)]_n$ Complexes: Kinetics and Mechanism. *Organometallics* **2012**, *31* (13), 4810–4816. <https://doi.org/10.1021/om300367k>.
51. Chang, S.; Waters, H.; Kettle, J.; Kuo, Z.; Li, C.; Yu, C.; Horie, M. Pd-Catalysed Direct Arylation Polymerisation for Synthesis of Low-Bandgap Conjugated Polymers and Photovoltaic Performance. *Macromol Rapid Commun* **2012**, *33* (22), 1927–1932. <https://doi.org/10.1002/marc.201200368>.
52. Zhang, X.; Shi, Y.; Dang, Y.; Liang, Z.; Wang, Z.; Deng, Y.; Han, Y.; Hu, W.; Geng, Y. Direct Arylation Polycondensation of β -Fluorinated Bithiophenes to Polythiophenes: Effect of Side Chains in C–Br Monomers. *Macromolecules* **2022**, *55* (18), 8095–8105. <https://doi.org/10.1021/acs.macromol.2c01266>.
53. Gobalasingham, N. S.; Thompson, B. C. Direct Arylation Polymerization: A Guide to Optimal Conditions for Effective Conjugated Polymers. *Prog Polym Sci* **2018**, *83*, 135–201. <https://doi.org/10.1016/j.progpolymsci.2018.06.002>.
54. Liang, Z.; Neshchadin, A.; Zhang, Z.; Zhao, F.-G.; Liu, X.; Yu, L. Stille Polycondensation: A Multifaceted Approach towards the Synthesis of Polymers with Semiconducting Properties. *Polym Chem* **2023**, *14* (40), 4611–4625. <https://doi.org/10.1039/D3PY00815K>.
55. Yu, J.; Rong, Y.; Kuo, C.-T.; Zhou, X.-H.; Chiu, D. T. Recent Advances in the Development of Highly Luminescent Semiconducting Polymer Dots and Nanoparticles for Biological Imaging and Medicine. *Anal Chem* **2017**, *89* (1), 42–56. <https://doi.org/10.1021/acs.analchem.6b04672>.

56. Kuo, C.-T.; Thompson, A. M.; Gallina, M. E.; Ye, F.; Johnson, E. S.; Sun, W.; Zhao, M.; Yu, J.; Wu, I.-C.; Fujimoto, B.; DuFort, C. C.; Carlson, M. A.; Hingorani, S. R.; Paguirigan, A. L.; Radich, J. P.; Chiu, D. T. Optical Painting and Fluorescence Activated Sorting of Single Adherent Cells Labelled with Photoswitchable Pdots. *Nat Commun* **2016**, 7 (1), 11468. <https://doi.org/10.1038/ncomms11468>.
57. Wu, C.; Schneider, T.; Zeigler, M.; Yu, J.; Schiro, P. G.; Burnham, D. R.; McNeill, J. D.; Chiu, D. T. Bioconjugation of Ultrabright Semiconducting Polymer Dots for Specific Cellular Targeting. *J Am Chem Soc* **2010**, 132 (43), 15410–15417. <https://doi.org/10.1021/ja107196s>.
58. Moon, J. H.; MacLean, P.; McDaniel, W.; Hancock, L. F. Conjugated Polymer Nanoparticles for Biochemical Protein Kinase Assay. *Chemical Communications* **2007**, No. 46, 4910. <https://doi.org/10.1039/b710807a>.
59. Sun, K.; Tang, Y.; Li, Q.; Yin, S.; Qin, W.; Yu, J.; Chiu, D. T.; Liu, Y.; Yuan, Z.; Zhang, X.; Wu, C. *In Vivo* Dynamic Monitoring of Small Molecules with Implantable Polymer-Dot Transducer. *ACS Nano* **2016**, 10 (7), 6769–6781. <https://doi.org/10.1021/acsnano.6b02386>.
60. Feng, L.; Zhu, C.; Yuan, H.; Liu, L.; Lv, F.; Wang, S. Conjugated Polymer Nanoparticles: Preparation, Properties, Functionalization and Biological Applications. *Chem Soc Rev* **2013**, 42 (16), 6620. <https://doi.org/10.1039/c3cs60036j>.
61. Grey, J. K.; Kim, D. Y.; Norris, B. C.; Miller, W. L.; Barbara, P. F. Size-Dependent Spectroscopic Properties of Conjugated Polymer Nanoparticles. *J Phys Chem B* **2006**, 110 (51), 25568–25572. <https://doi.org/10.1021/jp065990a>.
62. Guo, B.; Sheng, Z.; Kenry, K.; Hu, D.; Lin, X.; Xu, S.; Liu, C.; Zheng, H.; Liu, B. Biocompatible Conjugated Polymer Nanoparticles for Highly Efficient Photoacoustic Imaging of Orthotopic Brain Tumors in the Second Near-Infrared

- Window. *Mater. Horiz.* **2017**, 4 (6), 1151–1156.
<https://doi.org/10.1039/C7MH00672A>.
63. Ye, F.; Wu, C.; Jin, Y.; Wang, M.; Chan, Y.-H.; Yu, J.; Sun, W.; Hayden, S.; Chiu, D. T. A Compact and Highly Fluorescent Orange-Emitting Polymer Dot for Specific Subcellular Imaging. *Chemical Communications* **2012**, 48 (12), 1778. <https://doi.org/10.1039/c2cc16486h>.
64. Sarrazin, P.; Chaussy, D.; Vurth, L.; Stephan, O.; Beneventi, D. Surfactant (TTAB) Role in the Preparation of 2,7-Poly(9,9-Dialkylfluorene- Co - Fluorenone) Nanoparticles by Miniemulsion. *Langmuir* **2009**, 25 (12), 6745–6752. <https://doi.org/10.1021/la900259x>.
65. Ye, F.; Sun, W.; Zhang, Y.; Wu, C.; Zhang, X.; Yu, J.; Rong, Y.; Zhang, M.; Chiu, D. T. Single-Chain Semiconducting Polymer Dots. *Langmuir* **2015**, 31 (1), 499–505. <https://doi.org/10.1021/la5038684>.
66. Dai, C.; Pan, Y.; Liu, B. Conjugated Polymer Nanomaterials for Solar Water Splitting. *Adv Energy Mater* **2020**, 10 (42).
<https://doi.org/10.1002/aenm.202002474>.
67. Deng, S.; Li, L.; Zhang, J.; Wang, Y.; Huang, Z.; Chen, H. Semiconducting Polymer Dots for Point-of-Care Biosensing and In Vivo Bioimaging: A Concise Review. *Biosensors (Basel)* **2023**, 13 (1), 137.
<https://doi.org/10.3390/bios13010137>.
68. Frangioni, J. In Vivo Near-Infrared Fluorescence Imaging. *Curr Opin Chem Biol* **2003**, 7 (5), 626–634. <https://doi.org/10.1016/j.cbpa.2003.08.007>.
69. Rohatgi, C. V.; Harada, T.; Need, E. F.; Krasowska, M.; Beattie, D. A.; Dickenson, G. D.; Smith, T. A.; Kee, T. W. Low-Bandgap Conjugated Polymer Dots for Near-Infrared Fluorescence Imaging. *ACS Appl Nano Mater* **2018**, 1 (9), 4801–4808. <https://doi.org/10.1021/acsanm.8b01014>.

70. Kim, S.; Lim, C.-K.; Na, J.; Lee, Y.-D.; Kim, K.; Choi, K.; Leary, J. F.; Kwon, I. C. Conjugated Polymer Nanoparticles for Biomedical in Vivo Imaging. *Chemical Communications* **2010**, 46 (10), 1617. <https://doi.org/10.1039/b923309a>.
71. Liu, Y.; Liu, J.; Chen, D.; Wang, X.; Liu, Z.; Liu, H.; Jiang, L.; Wu, C.; Zou, Y. Quinoxaline-Based Semiconducting Polymer Dots for in Vivo NIR-II Fluorescence Imaging. *Macromolecules* **2019**, 52 (15), 5735–5740. <https://doi.org/10.1021/acs.macromol.9b01142>.
72. Massey, M.; Wu, M.; Conroy, E. M.; Algar, W. R. Mind Your P's and Q's: The Coming of Age of Semiconducting Polymer Dots and Semiconductor Quantum Dots in Biological Applications. *Curr Opin Biotechnol* **2015**, 34, 30–40. <https://doi.org/10.1016/j.copbio.2014.11.006>.
73. Chan, Y.-H.; Ye, F.; Gallina, M. E.; Zhang, X.; Jin, Y.; Wu, I.-C.; Chiu, D. T. Hybrid Semiconducting Polymer Dot–Quantum Dot with Narrow-Band Emission, Near-Infrared Fluorescence, and High Brightness. *J Am Chem Soc* **2012**, 134 (17), 7309–7312. <https://doi.org/10.1021/ja3022973>.
74. Wan, H.; Yue, J.; Zhu, S.; Uno, T.; Zhang, X.; Yang, Q.; Yu, K.; Hong, G.; Wang, J.; Li, L.; Ma, Z.; Gao, H.; Zhong, Y.; Su, J.; Antaris, A. L.; Xia, Y.; Luo, J.; Liang, Y.; Dai, H. A Bright Organic NIR-II Nanofluorophore for Three-Dimensional Imaging into Biological Tissues. *Nat Commun* **2018**, 9 (1), 1171. <https://doi.org/10.1038/s41467-018-03505-4>.
75. Ding, D.; Liu, J.; Feng, G.; Li, K.; Hu, Y.; Liu, B. Bright Far-Red/Near-Infrared Conjugated Polymer Nanoparticles for In Vivo Bioimaging. *Small* **2013**, 9 (18), 3093–3102. <https://doi.org/10.1002/smll.201300171>.
76. Zhang, X.; Yu, J.; Rong, Y.; Ye, F.; Chiu, D. T.; Uvdal, K. High-Intensity near-IR Fluorescence in Semiconducting Polymer Dots Achieved by Cascade FRET Strategy. *Chem Sci* **2013**, 4 (5), 2143. <https://doi.org/10.1039/c3sc50222h>.

77. Liu, H.-Y.; Wu, P.-J.; Kuo, S.-Y.; Chen, C.-P.; Chang, E.-H.; Wu, C.-Y.; Chan, Y.-H. Quinoxaline-Based Polymer Dots with Ultrabright Red to Near-Infrared Fluorescence for In Vivo Biological Imaging. *J Am Chem Soc* **2015**, *137* (32), 10420–10429. <https://doi.org/10.1021/jacs.5b06710>.
78. Liu, Y. , Y. J. , C. V. , & Z. Y. Conjugated Polymers for NIR-I and NIR-II Imaging. *General Chemistry* **2018**, *4* (170021–170021).
79. Foty, R. A Simple Hanging Drop Cell Culture Protocol for Generation of 3D Spheroids. *Journal of Visualized Experiments* **2011**, No. 51. <https://doi.org/10.3791/2720>.
80. Zanoni, M.; Piccinini, F.; Arienti, C.; Zamagni, A.; Santi, S.; Polico, R.; Bevilacqua, A.; Tesei, A. 3D Tumor Spheroid Models for in Vitro Therapeutic Screening: A Systematic Approach to Enhance the Biological Relevance of Data Obtained. *Sci Rep* **2016**, *6* (1), 19103. <https://doi.org/10.1038/srep19103>.
81. Walenta, S.; Doetsch, J.; Mueller-Klieser, W.; Kunz-Schughart, L. A. Metabolic Imaging in Multicellular Spheroids of Oncogene-Transfected Fibroblasts. *Journal of Histochemistry & Cytochemistry* **2000**, *48* (4), 509–522. <https://doi.org/10.1177/002215540004800409>.
82. Tanaka, K.; Mihara, T.; Koide, N. Synthesis and Physical Properties of Regioregular Poly(3-Alkoxy-4-Methylthiophene)s. *Polym J* **2004**, *36* (8), 628–633. <https://doi.org/10.1295/polymj.36.628>.
83. Yamashita, Y.; Ono, K.; Tomura, M.; Tanaka, S. Synthesis and Properties of Benzobis(Thiadiazole)s with Nonclassical π -Electron Ring Systems. *Tetrahedron* **1997**, *53* (29), 10169–10178. [https://doi.org/10.1016/S0040-4020\(97\)00356-6](https://doi.org/10.1016/S0040-4020(97)00356-6).
84. Eren, M. Ç.; Eren, A.; Dartar, S.; Kaya, B. U.; Üçüncü, M.; Varlikli, C.; Karakaya, H. Ç.; Emrullahoğlu, M. A Reaction-Based Scenario for Fluorescence

- Probing of Au(ⁱⁱⁱ) Ions in Human Cells and Plants. *Org Biomol Chem* **2023**, 21 (39), 7880–7885. <https://doi.org/10.1039/D3OB01081C>.
85. Mishriki, S.; Aithal, S.; Gupta, T.; Sahu, R. P.; Geng, F.; Puri, I. K. Fibroblasts Accelerate Formation and Improve Reproducibility of 3D Cellular Structures Printed with Magnetic Assistance. *Research* **2020**, 2020. <https://doi.org/10.34133/2020/3970530>.
 86. Kaminari, A.; Nikoli, E.; Athanasopoulos, A.; Sakellis, E.; Sideratou, Z.; Tsiourvas, D. Engineering Mitochondriotropic Carbon Dots for Targeting Cancer Cells. *Pharmaceuticals* **2021**, 14 (9), 932. <https://doi.org/10.3390/ph14090932>.
 87. Mao, Q.; Meng, Y.; Feng, Y.; Li, H.; Ma, T. Organelle Imaging with Carbon Dots: Strategies, Challenges, and Perspectives. *Inorg Chem Front* **2024**, 11 (3), 713–734. <https://doi.org/10.1039/D3QI02145A>.
 88. Zhang, Y.; Qiao, L.-L.; Zhang, Z.-Q.; Liu, Y.-F.; Li, L.-S.; Shen, H.; Zhao, M.-X. A Mitochondrial-Targetable Fluorescent Probe Based on High-Quality InP Quantum Dots for the Imaging of Living Cells. *Mater Des* **2022**, 219, 110736. <https://doi.org/10.1016/j.matdes.2022.110736>.
 89. Chakraborty, S.; Agrawalla, B. K.; Stumper, A.; Vegi, N. M.; Fischer, S.; Reichardt, C.; Kögler, M.; Dietzek, B.; Feuring-Buske, M.; Buske, C.; Rau, S.; Weil, T. Mitochondria Targeted Protein-Ruthenium Photosensitizer for Efficient Photodynamic Applications. *J Am Chem Soc* **2017**, 139 (6), 2512–2519. <https://doi.org/10.1021/jacs.6b13399>.
 90. Luo, S.; Tan, X.; Fang, S.; Wang, Y.; Liu, T.; Wang, X.; Yuan, Y.; Sun, H.; Qi, Q.; Shi, C. Mitochondria-Targeted Small-Molecule Fluorophores for Dual Modal Cancer Phototherapy. *Adv Funct Mater* **2016**, 26 (17), 2826–2835. <https://doi.org/10.1002/adfm.201600159>.

91. Horton, K. L.; Stewart, K. M.; Fonseca, S. B.; Guo, Q.; Kelley, S. O. Mitochondria-Penetrating Peptides. *Chem Biol* **2008**, *15* (4), 375–382. <https://doi.org/10.1016/j.chembiol.2008.03.015>.
92. Jung, H. S.; Lee, J.-H.; Kim, K.; Koo, S.; Verwilt, P.; Sessler, J. L.; Kang, C.; Kim, J. S. A Mitochondria-Targeted Cryptocyanine-Based Photothermogenic Photosensitizer. *J Am Chem Soc* **2017**, *139* (29), 9972–9978. <https://doi.org/10.1021/jacs.7b04263>.
93. Murphy, M. P.; Smith, R. A. J. Targeting Antioxidants to Mitochondria by Conjugation to Lipophilic Cations. *Annu Rev Pharmacol Toxicol* **2007**, *47* (1), 629–656. <https://doi.org/10.1146/annurev.pharmtox.47.120505.105110>.

VITA

EDUCATION

Ph.D. in Chemistry, Izmir Institute of Technology (2024)

M.Sc. in Chemistry, Izmir Institute of Technology (2020)

B.Sc. in Chemistry, Izmir Institute of Technology (2017)

ACADEMIC EXPERIENCE

- TÜBİTAK (The scientific and Technological Research Council of Türkiye) Project 01.2018 – 11.2018, Synthesis and Characterization of Conjugated Polyelectrolytes Sensitive to Conformation Change
- TÜBİTAK Project 01.2019 – 05.2021, Synthesis, characterization, and use of new generation paramagnetic agents with Lysine and Polyethylene glycol structured Gadolinium functionalized in cell culture using magnetic levitation method.
- TÜBİTAK Project 05.2021 – 02.2023, Synthesis of radiating Polymer-Dots (Pdot) in the near-infrared-II (NIR-II) region and Investigation of Penetration Behavior in Tumoroid Models
- TÜBİTAK Project 06.2023 – 07.2024, Study of the Penetration Systematics of Hydrophobic/Hydrophilic Thiophene Conjugated Copolymer Dots in the Artificial Blood-Brain Barrier Model.

SCHOLARSHIPS

- TÜBİTAK-BİDEB-2250-Graduate Scholarships Performance Program
- TÜBİTAK-BİDEB-2211-A General Domestic PhD Scholarship Program
- Council of Higher Education (CoHE) 100/2000 Scholarship

PUBLICATIONS

1. Karabacak, S. (2020). Synthesis of polythiophene-polyurethane soft nanoparticles for bioimaging applications (Ms Thesis, Izmir Institute of Technology (Turkey)).
2. Ammanath, G., Delachi, C. G., Karabacak, S., Ali, Y., Boehm, B. O., Yildiz, U. H., ... & Liedberg, B. (2021). Colorimetric and fluorometric profiling of advanced glycation end products. *ACS Applied Materials & Interfaces*, 14(1), 94-103.
3. Karabacak, S., Palaniappan, A., Tony, T. S. H., Edwin, T. H. T., Gulyás, B., Padmanabhan, P., & Yildiz, Ü. H. (2022). Gadolinium and polythiophene functionalized polyurea polymer dots as fluoromagnetic nanoprobes. *Nanomaterials*, 12(4), 642.
4. Karabacak, S., Qun, D. L. C., Ammanath, G., Yeasmin, S., Yagmurcukardeş, M., Alagappan, P., ... & Yıldız, Ü. H. (2023). Polarity induced vapochromism and vapoluminescence of polythiophene derivatives for volatile organic compounds classification. *Sensors and Actuators B: Chemical*, 389, 133884.
5. İnanç, D. Ö., Mutlu, M. U., Karabacak, S., & Yıldız, Ü. H. (2023). Advances and Future Perspective of Graphene Field Effect Transistors (GFETs) for Medical Diagnostics and Point-of-Care Tools. In *EMERGING TECHNOLOGIES IN BIOPHYSICAL SCIENCES: A WORLD SCIENTIFIC REFERENCE: Volume 3: Emerging Technologies for Diagnostics* (pp. 91-127).
6. Karabacak, S., Çoban, B., Yildiz A. A., Yildiz U. H. (2024) Near Infrared Emissive Super Penetrating Conjugated Polymer Dots for Intratumoral Imaging in 3D Tumor Spheroid Models. *Advanced Science*, 2403398.
7. Özefe, F., Karabacak, S., Yildiz U. H., Yildiz A. A. Synthesis and Characterization of New-Generation Paramagnetic Polymers and Their Use in Magnetic Levitation for Cell Sorting (in preparation)