

PhD Thesis

# *Development of Unnatural Lipids for the Delivery of DNA and Drug Molecules*

*A dissertation submitted to the  
Indian Institute of Technology Guwahati  
as partial fulfillment for the Degree of  
Doctor of Philosophy in Chemistry*

By  
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*To  
My Beloved Family*





# *Declaration*

6<sup>th</sup> June, 2022

I hereby declare that the thesis entitled **“Development of Unnatural Lipids for the Delivery of DNA and Drug Molecules”** is upshots of research that was carried out by me under the supervision of Prof. Debasis Manna, Department of Chemistry, Indian Institute of Technology Guwahati. This thesis has been submitted by me to the Department of Chemistry, Indian Institute of Technology Guwahati, for the award of the degree of Doctor of Philosophy. In keeping with the general practice of reporting scientific observations, due acknowledgments have been made wherever the work described is based on the findings of other investigators.

I further declare that this work has not been submitted anywhere else for any degree, diploma, associateship or membership etc., of any Institute or University to the best of my knowledge.



(Subhasis Dey)





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6<sup>th</sup> June, 2022

*To whom it may concern*

This is to certify that the thesis entitled **“Development of Unnatural Lipids for the Delivery of DNA and Drug Molecules”** is being submitted by Mr. Subhasis Dey (Roll No. 176122029) for the award of the PhD degree in Chemistry to the Indian Institute of Technology Guwahati, is a genuinely his own research work which was carried out by him. The information and data reported by him are completely his original findings. He has meticulously carried out the investigations and also followed the guidelines of my laboratory. Neither this thesis nor any part of it has been submitted for the award of any degree/diploma to anywhere before.

(Prof. Debasis Manna)



## *Contents*

|                      |     |
|----------------------|-----|
| Acknowledgements     | I   |
| Abstract             | III |
| List of Abbreviation | V   |
| Synopsis Report      | X   |

### **Chapter 1**

#### **Introduction of liposome-based DNA/drug delivery**

|                                                                                         |    |
|-----------------------------------------------------------------------------------------|----|
| 1.1. Introduction                                                                       | 1  |
| 1.2. Liposome as a DNA/drug delivery vehicle                                            | 2  |
| 1.3. Synthetic liposomal nanoparticles for DNA delivery                                 | 5  |
| 1.4. Structural evolution of the head, linker, and tail group of cationic lipid for DNA | 7  |
| 1.4.1. Design of cationic head group of lipid                                           | 8  |
| 1.4.2. Modification of the hydrophobic tail group                                       | 10 |
| 1.4.3. Modification of the linker moiety                                                | 11 |
| 1.5. Synthetic liposomal nanoparticles for anticancer drug delivery                     | 11 |
| 1.6. Stimuli-responsive anticancer drug delivery system                                 | 12 |
| 1.6.1. Internal Stimuli-Responsive drug delivery system                                 | 12 |
| 1.6.1.1. pH-Responsive delivery strategy                                                | 13 |
| 1.6.1.2. Redox-responsive delivery Strategy                                             | 14 |
| 1.6.2. NIR light-responsive liposome for drug delivery                                  | 16 |
| 1.7. Development of targeted and fluorescence liposome for theranostic application      | 19 |
| 1.8. Summary                                                                            | 20 |
| 1.9. References                                                                         | 21 |

### **Chapter 2**

#### **Lipid synthesis via sunlight mediated thiol-yne click reaction and DNA transfection efficiency**

|                                                                                                                                  |    |
|----------------------------------------------------------------------------------------------------------------------------------|----|
| 2.1. Background and objective of present work                                                                                    | 31 |
| 2.2. Results and discussions                                                                                                     | 32 |
| 2.2.1. Optimization of the reaction conditions for the thiol-yne click reactions under aqueous environment inhibitory activity a | 32 |
| 2.2.2. Synthesis of small molecules and cationic lipids                                                                          | 34 |
| 2.2.3. Morphology of the lipid vesicles                                                                                          | 36 |
| 2.2.4. Interaction of the lipids with the plasmid DNA                                                                            | 37 |
| 2.2.5. Zeta potential and hydrodynamic diameter of the lipid vesicles                                                            | 39 |
| 2.2.6. Phase transition temperature measurements                                                                                 | 41 |

|          |                                                                         |     |
|----------|-------------------------------------------------------------------------|-----|
| 2.2.7.   | Relation between the particle size and surface charge of the lipoplexes | 42  |
| 2.2.8.   | Cell viability and <i>in vitro</i> gene transfection efficiency         | 43  |
| 2.2.8.1. | Cell viability                                                          | 43  |
| 2.2.8.2  | Gene transfection efficiency of the lipoplexes                          | 44  |
| 2.3.     | Summary                                                                 | 46  |
| 2.4.     | Experimental section                                                    | 47  |
| 2.4.1.   | Synthesis of the compounds                                              | 47  |
| 2.4.1.1. | General information                                                     | 47  |
| 2.4.1.2. | General procedure for the thiol-ene/yne Click Reaction                  | 47  |
| 2.4.1.3. | General procedure for the synthesis of targeted compounds               | 48  |
| 2.4.2.   | Preparation of liposomes                                                | 59  |
| 2.4.3.   | Transmission electron microscopy imaging                                | 59  |
| 2.4.4.   | Particle size and surface change of the lipoplexes                      | 60  |
| 2.4.5.   | Isoelectric point measurements                                          | 60  |
| 2.4.6.   | Lipid phase-transition temperature measurement                          | 60  |
| 2.4.7.   | Preparation of lipoplexes                                               | 61  |
| 2.4.8.   | Gel retardation assay                                                   | 61  |
| 2.4.9.   | Cytotoxicity assay                                                      | 62  |
| 2.4.10.  | Measurement of transfection efficiency                                  | 62  |
| 2.4.11.  | GFP-uptake assays                                                       | 63  |
| 2.4.12.  | <sup>1</sup> H NMR and <sup>13</sup> C NMR of the compounds             | 64  |
| 2.4.13.  | Mass spectrum analysis of the intermediate of thio-yne click reaction   | 96  |
| 2.4.14.  | Substrate scopes for the synthesis of thioether compounds               | 97  |
| 2.4.15.  | Synthetic route to lipid headgroups                                     | 98  |
| 2.4.16.  | Gel retardation assay of the control compound                           | 98  |
| 2.4.17.  | TEM images of lipoplexes                                                | 99  |
| 2.4.18.  | FACS analysis for pMMP7-GFP uptake                                      | 100 |
| 2.4.19.  | Cellular viability of the lipids.                                       | 100 |
| 2.5.     | References                                                              | 101 |

### Chapter 3

#### Glutathione responsive anti-cancer drug delivery vehicle with inherent antibacterial activities

|        |                                                  |     |
|--------|--------------------------------------------------|-----|
| 3.1.   | Background and objective of present work         | 105 |
| 3.2.   | Results and discussions                          | 106 |
| 3.2.1. | Synthesis of sulfonium derivatives gemini lipids | 106 |
| 3.2.2. | Morphology of the lipid vesicles                 | 107 |
| 3.2.3. | Phase transition temperature measurement         | 108 |

|          |                                                                              |     |
|----------|------------------------------------------------------------------------------|-----|
| 3.2.4.   | Zeta potential and hydrodynamic diameter of the lipid vesicles               | 109 |
| 3.2.5.   | Glutathione mediated degradation of the lipid                                | 109 |
| 3.2.6.   | Drug loading and release efficiency of vesicle of 3.SL1a lipid               | 110 |
| 3.2.7.   | Cell viability and cellular uptake analysis                                  | 111 |
| 3.2.7.1. | Cell viability                                                               | 111 |
| 3.2.7.1. | Cellular uptake                                                              | 113 |
| 3.2.8.   | Antibacterial study                                                          | 114 |
| 3.3.     | Summary                                                                      | 115 |
| 3.4.     | Experimental section                                                         | 116 |
| 3.4.1.   | Synthesis of the compounds                                                   | 116 |
| 3.4.1.1. | General information                                                          | 116 |
| 3.4.1.2. | General procedure for the synthesis of targeted compounds                    | 116 |
| 3.4.2.   | Preparation of the lipid vesicles                                            | 123 |
| 3.4.3.   | Transmission electron microscopy analysis                                    | 124 |
| 3.4.4.   | Lipid phase transition temperature measurements                              | 124 |
| 3.4.5.   | pH-dependent particle size and surface potential measurement of the liposome | 125 |
| 3.4.6.   | Dealkylation of 3.SL1a lipid in the presence of glutathione                  | 125 |
| 3.4.7.   | Doxorubicin encapsulation and release efficiency measurement                 | 126 |
| 3.4.8.   | Cell culture                                                                 | 127 |
| 3.4.9.   | Determination of cell viability using MTT assay                              | 127 |
| 3.4.10.  | Determination of cellular uptake using microscopy analysis                   | 128 |
| 3.4.11.  | Determination of cellular uptake using flow cytometry                        | 128 |
| 3.4.12.  | Measurement of antibacterial activities                                      | 129 |
| 3.4.13.  | Morphological change analysis of the bacterial cells                         | 129 |
| 3.4.14.  | <sup>1</sup> H NMR and <sup>13</sup> C NMR spectra of synthesized compounds  | 130 |
| 3.4.15.  | TEM-EDX analysis of the lipid vesicle before and after glutathione treatment | 140 |
| 3.4.16.  | Size distribution analysis of the lipid vesicle                              | 140 |
| 3.4.17.  | Mass spectrum analysis of the glutathione mediated reaction of the lipid     | 141 |
| 3.4.18.  | Cytotoxicity analysis                                                        | 142 |
| 3.4.19.  | Antibacterial activity of the lipid against <i>E.coli</i> .                  | 142 |
| 3.5.     | References                                                                   | 143 |

## Chapter 4

### Development of photoresponsive macrocyclic lipid for anti-cancer drug delivery

|          |                                                                          |     |
|----------|--------------------------------------------------------------------------|-----|
| 4.1.     | Background and objective of present work                                 | 148 |
| 4.2.     | Results and discussions                                                  | 149 |
| 4.2.1.   | Synthesis of photoresponsive macrocyclic lipids                          | 149 |
| 4.2.2.   | Critical aggregation concentration (CAC) of the lipid                    | 150 |
| 4.2.3.   | Morphology of the lipid vesicles                                         | 150 |
| 4.2.4.   | Zeta potential and hydrodynamic diameter of the lipid nanoaggregates     | 151 |
| 4.2.5.   | Phase transition temperature measurements                                | 152 |
| 4.2.6.   | NIR light-induced photo cleavage of the compound Redox                   | 152 |
| 4.2.7.   | Drug loading and release efficiency of vesicle of <b>4.4.nIR12</b> lipid | 154 |
| 4.2.8.   | Cell viability and cellular uptake analysis                              | 155 |
| 4.2.8.1. | Cell viability                                                           | 155 |
| 4.2.8.2. | Cellular uptake                                                          | 156 |
| 4.3.     | Summary                                                                  | 157 |
| 4.4.     | Experimental section                                                     | 157 |
| 4.4.1.   | Synthesis of the compounds                                               | 157 |
| 4.4.1.1. | General information                                                      | 157 |
| 4.4.1.2. | General procedure for the synthesis of targeted compounds                | 158 |
| 4.4.2.   | Preparation of soluble aggregates                                        | 162 |
| 4.4.3.   | Transmission electron microscopy analysis                                | 162 |
| 4.4.4.   | Phase transition temperature measurements                                | 163 |
| 4.4.5.   | pH-dependent particle size and surface charge of the soluble aggregates  | 163 |
| 4.4.6.   | NIR light-induced cleavage of the amphiphile                             | 164 |
| 4.4.7.   | Doxorubicin encapsulation and release efficiency measurement             | 164 |
| 4.4.8.   | Cell culture                                                             | 164 |
| 4.4.9.   | Determination of cell viability using MTT assay                          | 165 |
| 4.4.10.  | Determination of the cellular uptake efficiency using flow cytometry     | 165 |
| 4.4.11.  | Determination of cellular uptake using microscopy analysis               | 166 |
| 4.4.12.  | $^1\text{H}$ and $^{13}\text{C}$ NMR spectra of the synthesized compound | 167 |
| 4.4.13.  | Purity analysis of the final lipid molecules                             | 172 |
| 4.4.14.  | Mass analysis of light-mediated cleavage                                 | 173 |
| 4.4.15.  | TEM images of the lipid after and before NIR light irradiation           | 173 |
| 4.4.16.  | Cell viability analysis                                                  | 174 |
| 4.4.17.  | Flow cytometry analysis                                                  | 175 |
| 4.5.     | References                                                               | 175 |



## Chapter 5

### Development of photoresponsive fluorescent lipid with cancer cell targetable ligand

|          |                                                                                  |     |
|----------|----------------------------------------------------------------------------------|-----|
| 5.1.     | Background and objective of present work                                         | 178 |
| 5.2.     | Results and discussions                                                          | 180 |
| 5.2.1.   | Synthesis of photoresponsive fluorescent lipids                                  | 180 |
| 5.2.2.   | Spectroscopic studies of the lipid 5.5.Cy3CAL                                    | 182 |
| 5.2.3.   | Morphological analysis of the vesicle of lipid 5.5.Cy3CAL                        | 183 |
| 5.2.4.   | Hydrodynamic diameter and zeta potential measurement of the lipid nanoaggregates | 184 |
| 5.2.5.   | Phase transition temperature measurements                                        | 185 |
| 5.2.6.   | NIR light-induced photo cleavage of the compound                                 | 185 |
| 5.2.7.   | Physical properties of NAs after light-mediated cleavage                         | 186 |
| 5.2.7.1. | Spectral properties analysis                                                     |     |
| 5.2.7.2. | Morphology analysis                                                              | 187 |
| 5.2.8.   | Drug loading and release efficiency of vesicle of 5.5.Cy3CAL lipid               | 188 |
| 5.2.9.   | Cell viability and cellular uptake analysis                                      | 190 |
| 5.2.9.1. | Cell viability                                                                   | 190 |
| 5.2.9.2. | Cellular uptake                                                                  | 191 |
| 5.3.     | Summary                                                                          | 192 |
| 5.4.     | Experimental section                                                             | 192 |
| 5.4.1.   | Synthesis of the compounds                                                       | 192 |
| 5.4.1.1. | General information                                                              | 192 |
| 5.4.1.2. | General procedure for the synthesis of targeted compounds                        | 193 |
| 5.4.2.   | Spectroscopic studies of the amphiphile                                          | 200 |
| 5.4.3.   | Preparation of soluble aggregates                                                | 200 |
| 5.4.4.   | Morphological analyses of the nano-aggregates                                    | 200 |
| 5.4.5.   | pH-dependent particle size and surface charge of the soluble aggregates          | 201 |
| 5.4.6.   | Phase transition temperature measurements                                        | 201 |
| 5.4.7.   | NIR light-induced cleavage of the amphiphile                                     | 201 |
| 5.4.8.   | Doxorubicin encapsulation and release efficiency measurement                     | 202 |
| 5.4.9.   | Cell culture                                                                     | 202 |
| 5.4.10.  | Determination of cell viability using MTT assay                                  | 203 |
| 5.4.11.  | Determination of cellular uptake efficiency using flow cytometry                 | 203 |
| 5.4.12.  | Determination of cellular uptake using microscopy analysis                       | 203 |
| 5.4.13.  | $^1\text{H}$ NMR and $^{13}\text{C}$ NMR spectra of synthesized compounds        | 205 |
| 5.4.14.  | Fluorescence based CAC measurement                                               | 217 |

|                             |     |
|-----------------------------|-----|
| 5.4.15. Cell viability      | 217 |
| 5.5. References             | 218 |
| 6. <b>Thesis Conclusion</b> | 222 |
| 7. <b>Future Prospects</b>  | 222 |
| <b>Annexure I</b>           | 224 |
| <b>Annexure II</b>          | 225 |
| <b>Publication</b>          | 226 |



## Acknowledgements

*This dissertation is a consequence of endless support and encouragement by numerous well-wishers'. I would like to acknowledge each of them for helping me to reach the milestone in my life.*

*Prior to all, I would like to convey my sincere gratitude to my supervising guide, Prof. Debasis Manna, who introduced me to the splendid world of Science. His consistent guidance, encouragement, and scholarly inputs enrich me a lot for shining in science. I am truly honored and blessed to have been a part of his research group. Apart from research, his thoughts and ideas about life paved the way for me to be a better person in the future. Thank you, Sir, for being my Mentor.*

*My sincere and heartfelt thanks to my collaborators, Dr. Sachin Kumar (Dept. of BSBE, IIT Guwahati, India), Dr. Rajkumar P. Thummer (Dept. of BSBE, IIT Guwahati, India), Prof. Arindam Bhattacharyya (Dept. of Zoology, University of Calcutta, India), and Prof. Dr. Siddhartha Sankar Ghosh (Dept. of BSBE, IIT Guwahati) for their constructive support in my research.*

*I would also like to thank my doctoral committee members Prof. Subhas Chandra Pan, Dr. Animesh Das, and Dr. Rajkumar P. Thummer, for their valuable suggestions and consistent evaluation of my research works.*

*I would like to express my gratitude to all the faculty and staff members of the Department of Chemistry for their continuous support. Also, I am immensely thankful to the Department of Chemistry and CIF (IIT Guwahati) for allowing me to use the sophisticated instrument facility. A special mention in this note of acknowledgment will certainly be of IIT Guwahati for providing me with the scholarship.*

*I would also like to acknowledge all my past and present lab members, including Dr. Sreeparna Das, Dr. Saurav Paul, Dr. Subhankar Panda, Dr. Ashalata Roy, Dr. Abhishek Saha, Mr. Dipjoyti Talukdar, Dr. Nirmalya Pradhan, Dr. Nasim Akhtar, Ms. Oindrila Biswas, Mr. Sribash Das, Ms. Anjali Patel, Ms. Priyanka Mazumder, Mr. Gunanka Hazarika, Mr. Biswa Mohan Prusty, Ms. Soumya Srimayee, Ms. Nikumoni Das and Mr. Mohit Kumar for providing me a healthy and friendly atmosphere in the lab.*

*I extend my sincere thanks to all of my friends for their constant support and encouragement.*

*Last but not least, my Ph. D. endeavors would not have been accomplished without acknowledging my loving family and parents, Ms. Lalita Dey and Mr. Sushil Dey, and my brother Snehasis Dey and my loved one, Susmita for tolerating me till now. I dedicate this page to the emotion of my life, my Late Grandmother and Grandfather, who will always be in my soul and memory. Their endless love, continuous support, and blessings boost me up to cross the hurdles of my career.*

*Thank you all for being a memorable part of my life.*

***Mr. Subhasis Dey***

## Abstract

The thesis entitled "***Development of Unnatural Lipids for the Delivery of DNA and drug Molecules***" demonstrates the synthesis, characterization, and biophysical studies of the functionalized unnatural lipid molecules and their applications in delivering DNA and drug molecules to the mammalian cells. The thesis has been arranged into five chapters based on their development and experimental results, which were carried out during the research period.

**Chapter 1** describes the necessity for the development of drug delivery systems (DDSs) and how synthetic lipid-based DDSs showed great potential in overcoming the low bioavailability of poorly aqueous soluble therapeutics molecules. This chapter also discusses how lipid or amphiphilic molecules can be tuned with different active and passive targetable functional groups for the delivery of bioactive components to the targeted sites.

**Chapter 2** demonstrate the development of the synthetic method for thiol-ene/yne click reaction under sunlight conditions in the aqueous medium to synthesize a series of  $\beta$ -hydroxy ammonium head groups containing amphiphilic molecules. The synthesized cationic lipid's detailed biophysical characterization and *in vitro* DNA transfection efficiency were also documented.

**Chapter 3** describes the development of a novel class of stimuli-sensitive sulfonium-based synthetic amphiphiles, which exhibit several favorable biophysical properties of natural lipids. The anticancer drug (doxorubicin (Dox)) delivery efficacy of the potent amphiphiles and its inherent antibacterial activities were also discussed.

**Chapter 4** reports a smart approach to delivering the anticancer drug. Here, developed NIR-light-responsive macrocyclic cationic gemini amphiphiles and examined the light-triggered Dox delivery efficacy to cancerous cells.

**Chapter 5** elaborates on developing a cyanine-3 (Cy3) dye-based fluorescent macrocyclic targetable amphiphile with NIR-light mediate cleavable property. The image-guided Dox delivery to the cancer cell has been reflected in this chapter.



### *List of Abbreviation*

|                   |                                                                        |
|-------------------|------------------------------------------------------------------------|
| ADME              | Absorption, distribution, metabolism, excretion                        |
| AIBN              | Azobisisobutyronitrile                                                 |
| BHK               | Baby hamster kidney                                                    |
| BSO               | Buthionine sulfoximine                                                 |
| CA-IX             | Carbonic anhydrases-IX                                                 |
| CLANs             | Cationic lipid-assisted nanoparticle                                   |
| Chol              | Cholesterol                                                            |
| CLSM              | Confocal laser scanning microscopy                                     |
| CuI               | Copper iodide                                                          |
| CAC               | Critical aggregation constant                                          |
| Cys               | Cysteine                                                               |
| Cy3               | Cyanine-3 dye                                                          |
| DDS               | Drug delivery system                                                   |
| CDCl <sub>3</sub> | Deuterated chloroform                                                  |
| DCM               | Dichloromethane                                                        |
| DIAD              | Diisopropyl azodicarboxylate                                           |
| DLS               | Dynamic light scattering                                               |
| DMF               | Dimethylformamide                                                      |
| DMEM              | Dulbecco's modified Eagle's medium                                     |
| DMSO              | Dimethyl sulfoxide                                                     |
| DODAC             | Dioleoyl-dimethyl-ammonium chloride                                    |
| DOPE              | 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine                          |
| DOTMA             | <i>N</i> -[1-(2,3-Dioleoyloxy)propyl]- <i>N,N,N</i> -trimethylammonium |
| DTBP              | Di-tert-butyl peroxide                                                 |
| Dox               | Doxorubicin                                                            |
| DNA               | Deoxyribonucleic acid                                                  |
| DLS               | Dynamic light scattering                                               |
| ESI-MS            | Electrospray ionization-mass spectrometry                              |
| $\lambda_{em}$    | Emission wavelength                                                    |

|                               |                                                            |
|-------------------------------|------------------------------------------------------------|
| EPR                           | Enhanced permeation and retention                          |
| <i>E. coli</i>                | Escherichia coli                                           |
| EtOAc                         | Ethyl acetate                                              |
| EDTA                          | Ethylenediaminetetraacetic acid                            |
| $\lambda_{\text{ex}}$         | Excitation wavelength                                      |
| FBS                           | Fetal Bovine Serum                                         |
| FDA                           | Food and drug administration                               |
| FESEM                         | Field emission scanning electron microscopy                |
| FETEM                         | Field emission transmission electron microscopy            |
| FACS                          | Fluorescence-activated cell sorting                        |
| GUV                           | giant unilamellar vesicle                                  |
| GSH                           | Glutathione                                                |
| IC <sub>50</sub>              | Half maximal inhibitory concentration                      |
| IVIVC                         | <i>in vitro-in vivo</i> correlation                        |
| HPLC                          | High-performance liquid chromatography                     |
| HRMS                          | High-resolution mass spectrometry                          |
| HEK-293T                      | Human embryonic kidney 293                                 |
| HCl                           | Hydrochloric acid                                          |
| d <sub>H</sub>                | Hydrodynamic diameters                                     |
| H <sub>2</sub> O <sub>2</sub> | Hydrogen peroxide                                          |
| HIF                           | Hypoxia-inducible factors                                  |
| IEP                           | Isoelectric point                                          |
| LiOH                          | Lithium hydroxide                                          |
| LNPs                          | Lipid nanoparticles                                        |
| LUVs                          | Large unilamellar vesicles                                 |
| MgCl <sub>2</sub>             | Magnesium chloride                                         |
| MALDI-TOF                     | Matrix assisted laser desorption ionization-time of flight |
| T <sub>m</sub>                | Phase transition temperature                               |
| MHz                           | Mega Hertz                                                 |
| MeOH                          | Methanol                                                   |
| MIC                           | Minimum inhibitory concentration                           |
| MLVs                          | Multilamellar vesicles                                     |
| NAs                           | Nano-aggregates                                            |



|                                 |                                             |
|---------------------------------|---------------------------------------------|
| nm                              | Nanometre                                   |
| NP                              | Nanoparticle                                |
| NBS                             | <i>N</i> -bromosuccinimide                  |
| NIR                             | Near infrared                               |
| DIPEA                           | <i>N,N</i> -Diisopropylethylamine           |
| N <sub>2</sub>                  | Nitrogen                                    |
| NMR                             | Nuclear magnetic resonance                  |
| OD                              | Optical density                             |
| O <sub>2</sub>                  | Oxygen                                      |
| PBMC                            | Peripheral blood mononuclear cell           |
| PBS                             | Phosphate-buffered saline                   |
| DPPC                            | Phospholipid dipalmitoylphosphatidylcholine |
| H <sub>3</sub> PO <sub>4</sub>  | Phosphoric acid                             |
| pMMP7                           | Plasmid matrix metalloproteinase-7          |
| PDI                             | Polydispersity indexes                      |
| PEG                             | Polyethylene Glycol                         |
| KCl                             | Potassium chloride                          |
| KOH                             | Potassium hydroxide                         |
| PI                              | Propidium iodide                            |
| PEG                             | Polyethylene glycol                         |
| ROS                             | <i>Reactive oxygen species</i>              |
| RBC                             | Red blood cells                             |
| RPM                             | Revolutions per minute                      |
| AgBF <sub>4</sub>               | Silver tetrafluoroborate                    |
| NaCl                            | Sodium chloride                             |
| SDS                             | Sodium dodecyl sulfate                      |
| NaH                             | Sodium hydride                              |
| NaBH <sub>4</sub>               | Sodium hydrogen boride                      |
| Na <sub>2</sub> SO <sub>4</sub> | Sodium sulfate                              |
| <i>S. aureus</i>                | <i>Staphylococcus aureus</i>                |
| SARs                            | Structure-activity relationship             |
| SUVs                            | Small unilamellar vesicles                  |
| TEMPO                           | (2,2,6,6-tetramethylpiperidin-1-yl)oxyl     |

|                  |                                                                          |
|------------------|--------------------------------------------------------------------------|
| THF              | Tetrahydrofuran                                                          |
| TMS              | Tetramethylsilane                                                        |
| TBAB             | Tetrabutylammonium bromide                                               |
| TBHP             | Tert-butyl hydroperoxide                                                 |
| TLC              | Thin layer chromatography                                                |
| TEM              | Transmission electron microscopy                                         |
| TEM-EDX          | Transmission electron microscopy-energy dispersive X-ray spectrometry    |
| TAE              | Tris-acetate                                                             |
| TFA              | Trifluoroacetic acid                                                     |
| Tris-HCl         | tris(hydroxymethyl)aminomethane-hydrochloric acid                        |
| TPA              | Two-photon absorption                                                    |
| UV-Vis           | Ultraviolet-visible                                                      |
| H <sub>2</sub> O | Water                                                                    |
| DPPS             | 1,2-Dipalmitoyl-sn-glycero-3-phosphoserine                               |
| DPH              | 1,6-diphenyl-1,3,5-hexatriene                                            |
| HBTU             | (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate |
| MTT              | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide            |
| MOPS             | 3-(N-morpholino)propanesulfonic acid                                     |
| HEPES            | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid                       |
| DAPI             | 4',6-diamidino-2-phenylindole                                            |

#### For symbols/units

|          |                   |
|----------|-------------------|
| $\alpha$ | Alpha             |
| Å        | Angstrom          |
| atm      | Atmosphere        |
| $\beta$  | Beta              |
| C        | Celsius           |
| J        | Coupling constant |
| °        | Degree            |
| $\delta$ | Delta             |
| Hz       | Hertz             |

---

|      |                                                 |
|------|-------------------------------------------------|
| K    | Kelvin                                          |
| MHz  | Megahertz                                       |
| m    | Meta                                            |
| µg   | Micro gram                                      |
| µM   | Micro mole                                      |
| mL   | Millilitre                                      |
| mV   | Mili Volt                                       |
| min  | Minute                                          |
| nM   | Nano mole                                       |
| o    | Ortho                                           |
| p    | Para                                            |
| ppm  | Parts per million                               |
| %    | Percent                                         |
| π    | Pi                                              |
| ps   | Pico second                                     |
| s    | Second                                          |
| ADME | Absorption, distribution, metabolism, excretion |



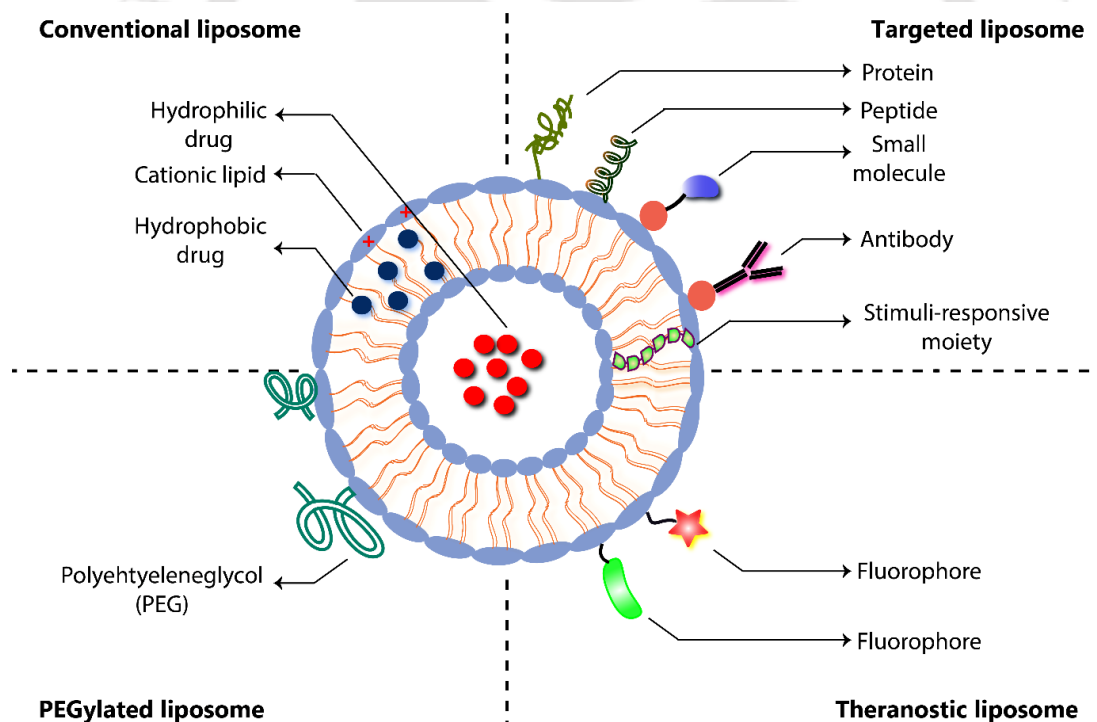
## Chapter 1

### Introduction

The evolution of new drug molecules or modification of drugs is always a considerable challenge. Pertinent delivery strategies have helped convert usual therapeutics (mainly hydrophobic bioactive molecules) into promising therapies.<sup>1-3</sup>. The therapeutic efficacy of exogenously administered bioactive molecules primarily relies on the extent of their prolonged availability in the desired site. The drug molecule depends on a few intrinsic factors related to the nature of the drug molecule itself, such as drug solubility (drug solubility: importance and enhancement techniques), pKa profile of the drug (The pKa distribution of drugs: application to drug discovery), acceptance by the receptor (The receptor concept: pharmacology's big idea), molecular weight (Molecular properties that influence oral drug-like behavior), among others.<sup>4</sup> These characteristics cause directly affect the cell membrane permeability of the molecules and, therefore, their ability to ingress into the target cell and produce its biological activity in it.

On the other hand, some extrinsic factors, such as the physiological stage of the receptor organism, enzymatic machinery, and external pH in the surrounding environment, make the drug prone to inactivation or degradation. Moreover, some other substances encountered throughout the organisms during the distribution process may interact with the drug in different ways, resulting in either inactivation by the formation of molecular complexes or synergistic interaction that may modulate the drug's potency or generate unexpected responses.<sup>5</sup> Manipulating with biophysical barrier, the drug delivery systems (DDSs) have been pursued vigorously to deliver the drug protectively to the targeted sites. As the therapeutic landscape evolved, delivery strategies and technologies quickly adapted to changing drug delivery needs. Due to extensive fundamental exploration in liposome technology, several liposome-based drug formulations are available for human use in different therapies, and several products are under different clinical trials.<sup>6</sup> Encapsulation of drug molecules in liposomes improved the therapeutic indices in various ways, mainly through alterations in their pharmacokinetics and pharmacodynamics. Therapeutics with different solubility can be entrapped in liposomes, hydrophobic molecules have an affinity to the phospholipid bilayer, and hydrophilic drugs are stabilized in the central aqueous cavity. To accomplish this point, the

pharmacological properties of liposome-based carriers have been greatly advanced perpetually through a significant amount of research aimed at optimizing the stable assembly, prolonged circulation time, and cell-specific targeting of liposomes. The advancement of the linker, hydrophobic part of the lipid molecules optimizes the stability of liposomes by diminishing recognition and decomposition by opsin proteins of the reticuloendothelial system (RES). In addition, the surface modification of the liposome with an appropriate functional group enhances the ability of the delivery genetic material to the cell; in another way, the functionalization of liposomes with a specific receptor, monoclonal antibodies, or antibody fragments to generate immune liposomes has emerged as a promising strategy for targeted delivery to and uptake by cells overexpressing the antigens to these antibodies. Immunoliposome has been harnessed to enhance selective delivery to diseased cells, thereby circumventing side effects.<sup>7</sup>



**Figure 1.1.** Modification of lipid molecules with different functional groups.

Nowadays, an emerging area of ongoing research involves the development of stimuli-responsive liposomes. This approach shows great prospects for enhancing drug release in-or-around diseased cells by exploiting endogenous stimuli (e.g., pH,

redox gradient, and enzyme concentration of the disease cell microenvironment) or exogenous stimuli (temperature, light, magnetic field, and ultrasound).<sup>8</sup> In addition, modifying the lipid carrier with a fluorophore for early diagnosis or image-guided drug delivery (IGDD) refers to the combination of drug targeting and imaging of preclinical and clinical applications of theranostic nanoplateforms, highlighting molecular imaging modalities and cancer cell detection strategies. Preclinically, IGDD can help by intraoperative guidance in monitoring the biodistribution profile, target site accumulation, off-target localization, drug release, and drug efficacy. However, clinically, it holds significant potential for preselecting patients. Hence, this biocompatible liposome system with diversified functionality combines diagnostic and therapeutic functions for highly efficient, safer anticancer treatments.

### **Aim of the Thesis**

In the present thesis, we have focused on developing smart amphiphilic molecules for targeted delivery. We have explored the delivery efficacy of our synthesized amphiphiles for the treatment of cancer. The entire work is arranged into four chapters, as given below.

In **chapter 2**, we have focused on how the mild synthetic strategy can also be used for the smart design of novel or improvement of prevailing lipid-based nonviral gene delivery systems.

**Chapter 3** mainly deals with how glutathione (GSH) responsive sulfonium-based lipid molecules successfully release the encapsulated drug molecules to the reductive tumor environment along with inherent antimicrobial activities against both Gram-positive and Gram-negative bacteria.

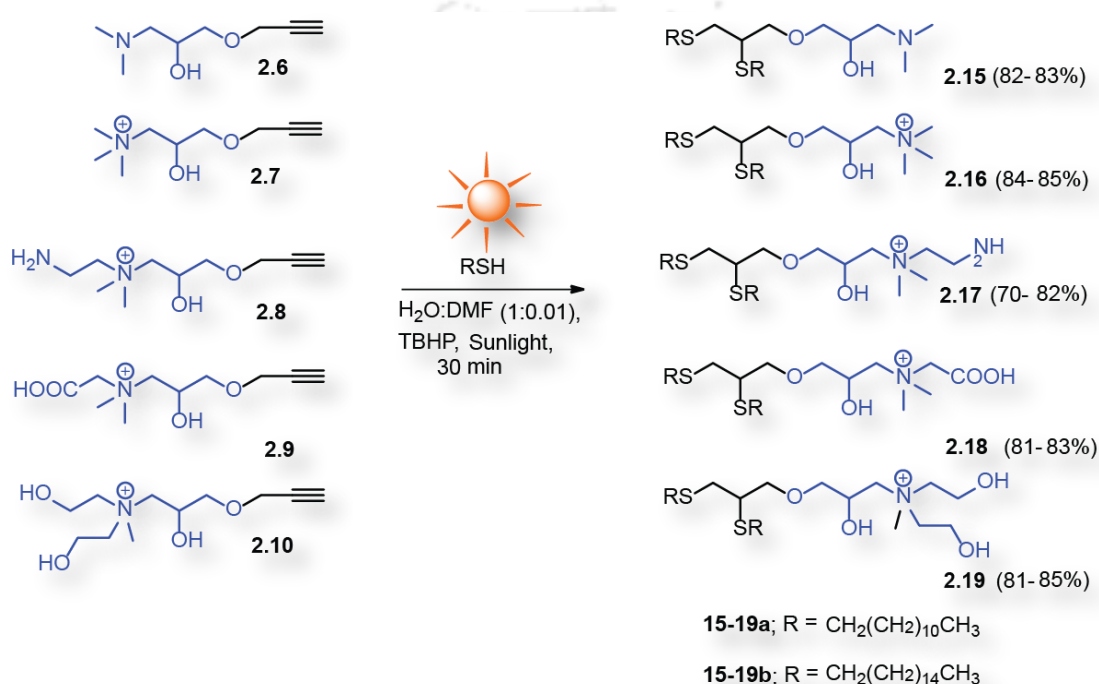
**Chapter 4** elaborates on how we developed NIR-light-responsive macrocyclic cationic gemini amphiphiles for anticancer drug delivery and light mediate transformation from spherical to nanotubular assemblies.

**Chapter 5** corroborates how we developed a single amphiphile that can short out the necessary conditions of smart DDS demands. We synthesized a photoresponsive fluorescent macrocyclic amphiphile with a cancer cell targeting ligand.

## Chapter 2

### Lipid synthesis via sunlight mediated thiol-yne click reaction and DNA transfection efficiency

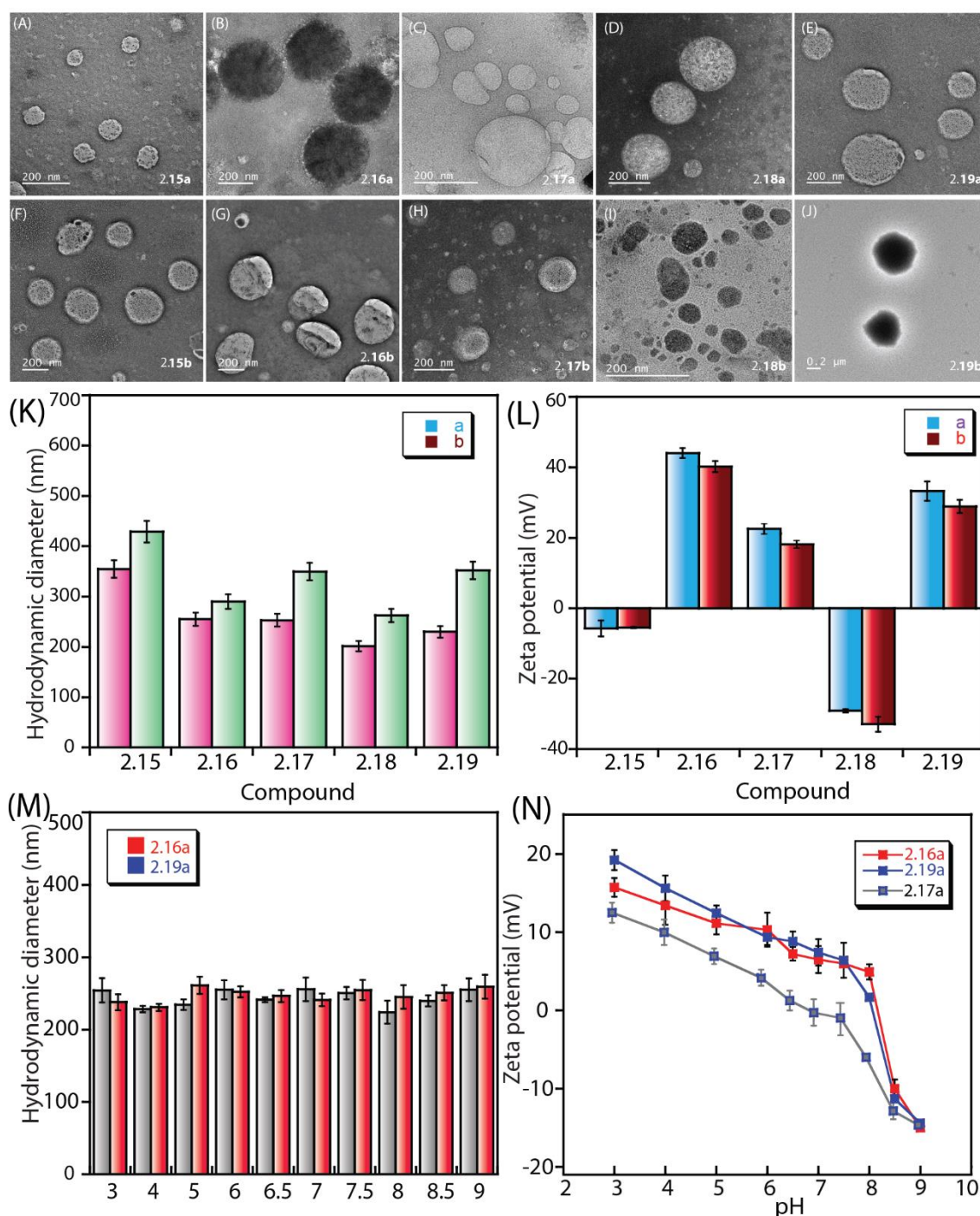
This study describes the development of a mild approach to thiol-ene/yne click reaction in an aqueous medium under sunlight. The optimized reaction conditions were successfully applied to synthesize a series of cationic lipids for DNA delivery. The amphiphilic molecules form spherical nanoaggregates (NAs) in water.



**Scheme 2.1** Synthesis of dithioether-based cationic lipids through sunlight-mediated thiol-yne clicks reactions in the aqueous medium.

We characterized the physicochemical properties such as particle size, surface potential, and phase-transition temperature of the amphiphiles. These biophysical studies confirmed the liposome likeliness property of the synthesized cationic lipids. The pH-dependent dynamic light scattering (DLS) measurements showed that the pH of the environment has little impact on the hydrodynamic diameter of the lipids (Figure 2.1K, M). However, the pH of the environment plays a vital role in the surface charge of the



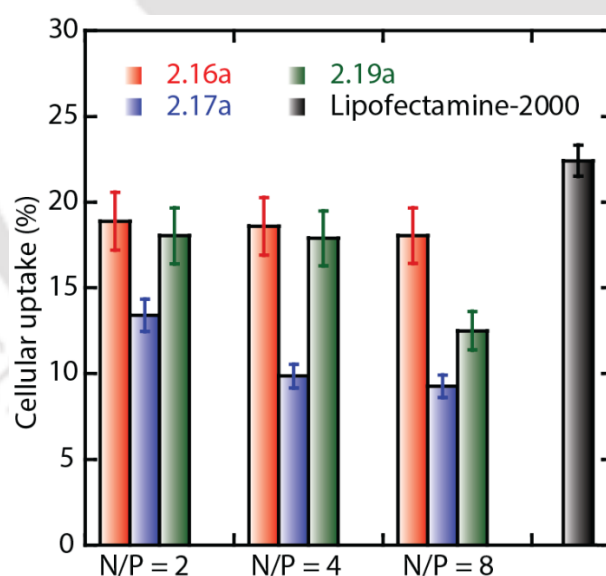


**Figure 2.1** TEM images of the lipids in an aqueous medium (A-J). Mean particle size of the lipid vesicles at pH 7.4 (K). Surface potential at pH 7.4 (L). The pH-dependent hydrodynamic diameter (M) and surface potential (N).

lipids, which could be due to the presence of ammonium ion in the lipid headgroup and three compounds showing positive surface potential at physiological pH (Figure 2.1L). Phase-transition temperature ( $T_m$ ) of the cationic lipids can be considered a fundamental physical property for investigating the liposomes' thermal stability and

gene delivery properties.  $T_m$  values of lipids **2.16a** and **2.19a** were 43 and 37 °C, respectively.

This elevation of  $T_m$  values could be due to the more ordered packing in the plane of the lipid bilayer. This moderate thermal stability also suggests that these lipids could have multiple biological applicability. DNA complexation ability of the lipids was analyzed by gel retardation assay, and the results confirmed that lipids **2.16**, **2.17**, and **2.19** strongly retards the mobility of the plasmid DNA by effective interaction with the  $\beta$ -hydroxy ammonium group with negatively charged DNA. Surprisingly, the lipid **2.20** with trimethylammonium headgroups instead of  $\beta$ -hydroxy ammonium moiety showed lower plasmid DNA-binding, suggesting the presence of the  $\beta$ -hydroxy group in the linker region plays a vital role in their DNA-binding abilities. The potent cationic lipids **2.16**, **2.17**, and **2.19** displayed much lower cytotoxicity. Interestingly, these cationic lipids also showed comparable gene transfection and cellular uptake properties with the commercially available Lipofectamine 2000 (Figure 2.2), signifying their prospective to be favorable nonviral gene vectors.

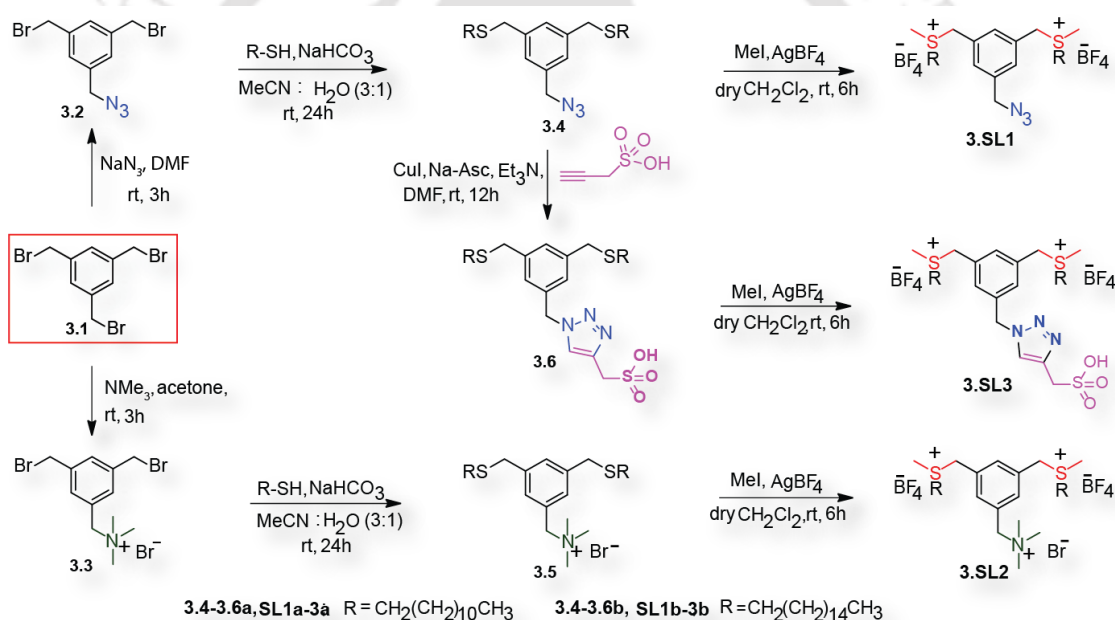


**Figure 2.2** The MMP7-GFP transfection efficiency (%) of the lipids at different N/P ratios in HEK-293T cells after 24 hours of transfection.

### Chapter 3

#### Development of stimuli-responsive sulfonium based cationic gemini amphiphile for anticancer drug delivery with inherent antibacterial activities

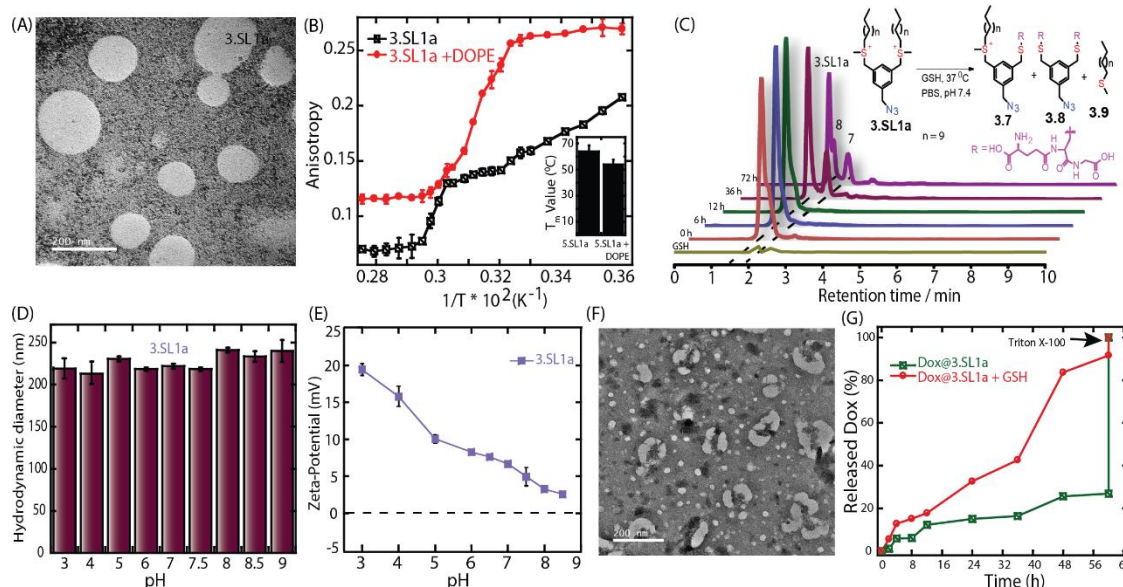
The delivery of drug molecules to specific cells or tissues through the blood circulation system without altering their pharmacokinetics and pharmacodynamics is one of the major challenges in the drug development process.<sup>9-11</sup> The stimuli-responsive DDSs have been considered one of the practical approaches to fight against cancer and other diseases.<sup>12-14</sup> In this study, we synthesized a series of novel sulfonium-based lipids, which showed a wide range of favorable biophysical properties such as the formation of aqueous soluble spherical aggregates and high phase transition temperature and stimuli-responsive properties.



**Scheme 3.1** Synthetic route of the sulfonium-based lipids.

The hydration of the dried film of the lipid ensued in the formation of colloidal aggregates. The analysis of transmission electron microscopy (TEM) images revealed the formation of spherical aggregates of the **3.SL1a** lipid. The dynamic light scattering (DLS) measurement revealed that the hydrodynamic diameters ( $d_H$ ) of the vesicles were within 200–250 nm, and the pH of the solution had a negligible effect on its  $d_H$  values (Figure 3.1D). The zeta-potential measurement at various pH values

revealed that the vesicles form a positive surface potential (Figure 3.1E), which could be due to the presence of sulfonium moieties. The temperature-dependent steady-state fluorescence anisotropy measurements revealed that the phase transition temperature ( $T_m$ ) of the **3.SL1a** lipid was 64 °C.

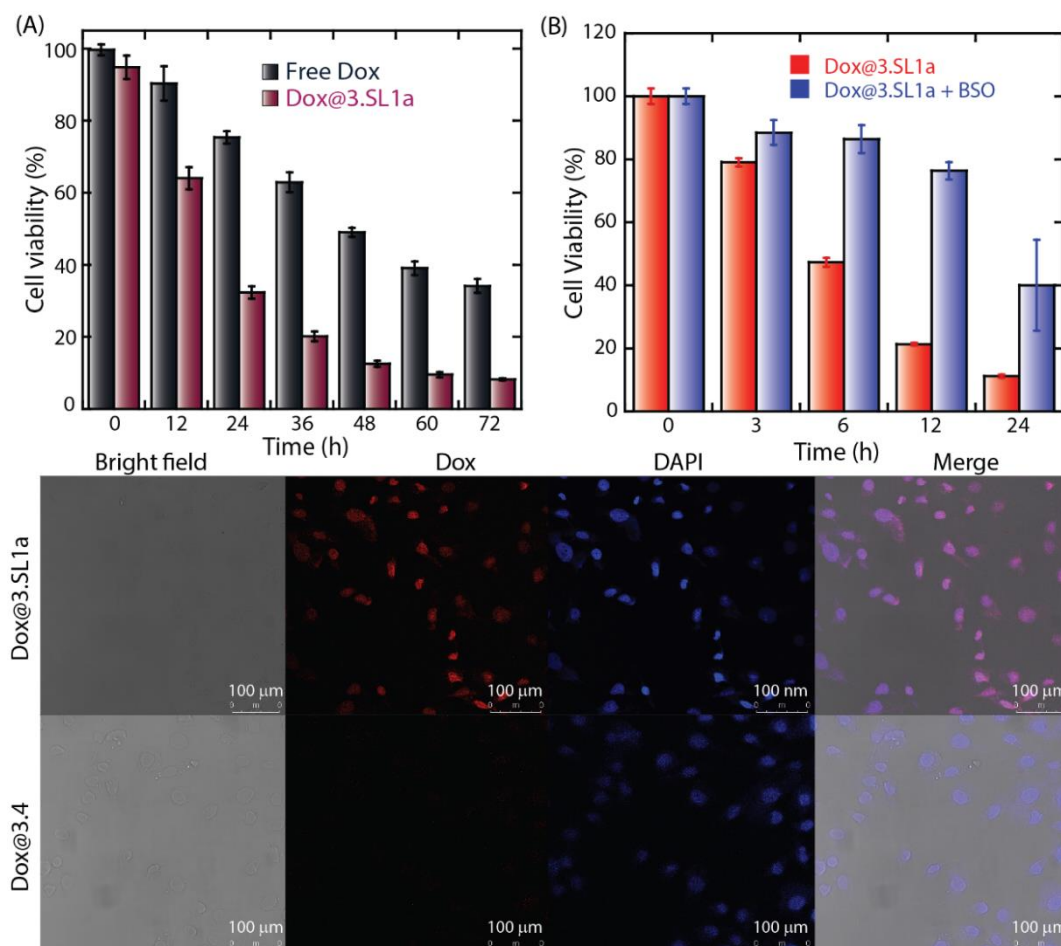


**Figure 3.1** TEM image of lipid **3.SL1a** (A). Phase transition temperature (B). Time-dependent HPLC analysis of **3.SL1a** NAs in 10 mM GSH (C). The pH-dependent DLS and zeta potential measurement (D and E). **3.SL1a** morphology in 10 mM GSH environment (F). Dox release profile from NAs of **3.SL1a** in the presence and absence of GSH (G)

GSH-responsive property of the sulfonium groups investigates whether the vesicles can be deformed in a controlled manner in the presence of stimuli GSH. To confirm the dealkylation of the **3.SL1a** lipid, we performed the HPLC analysis in the absence and presence of GSH (10 mM) in PBS (pH 7.4) at 37 °C (Figure 3.1C). The time-dependent HPLC analysis revealed that the formation of mono- and di-dealkylated intermediates start after 36 and 72 h of incubation (Figure 3.1C). The control experiment showed that the **3.SL1a** lipid is very much stable in the absence of GSH under similar experimental conditions. The TEM and TEM-EDX analysis of the **3.SL1a** lipid in the presence of GSH also showed a significant change in the vesicles' aggregation arrangements, indicating its instability under the reduced environment. Anticancer drug Dox loading capacity of these NAs was observed 41%; however, a faster release profile of Dox from the vehicle was confirmed in the presence of a GSH



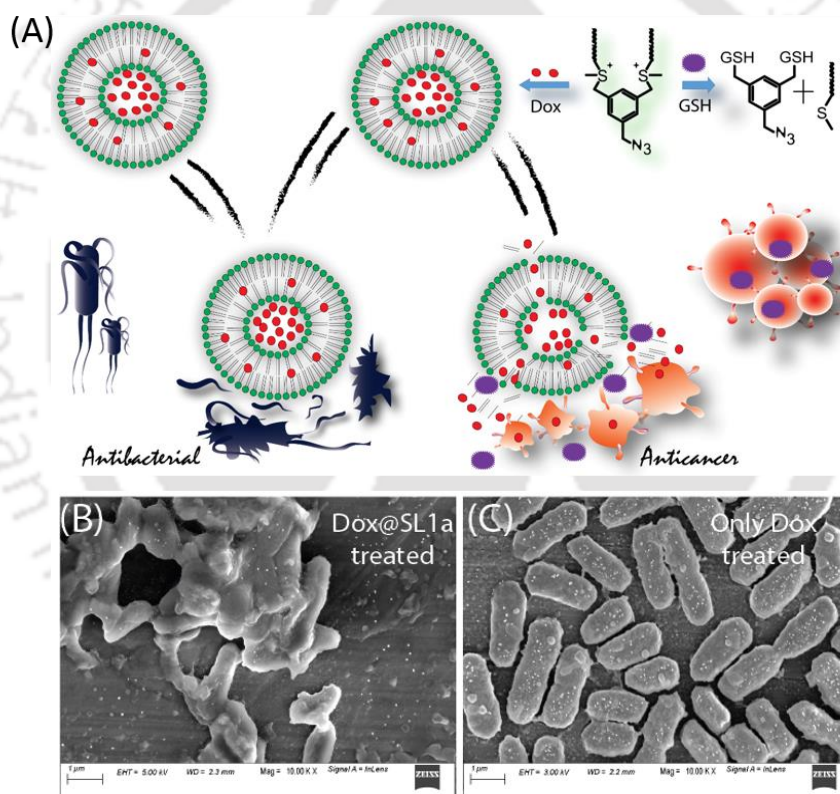
environment (Figure 3.1G). Additionally, the potent lipid has lower cytotoxicity and higher efficiency in encapsulating and releasing the hydrophobic drug doxorubicin (Dox) to cancer cells. The cellular uptake efficiency in the absence and presence of a GSH inhibitor demonstrated that the intracellular GSH level acts as a stimulus to release the drug molecules into cancer cells.



**Figure 3.2** Time-dependent viabilities of MDA-MB-231 cells in the presence of free Dox and Dox@3.SL1a at 0.36 and 0.49 μM, respectively (A). Viabilities of the Dox@3.SL1a (0.49 μM) treated MDA-MB-231 cells in the absence and presence of a GSH inhibitor, BSO (1 mM) (B). CLSM images of the MDA-MB-231 cells treated with Dox@3.SL1a (0.49 μM) for 8 h (C).

The presence of sulfonium moieties and recent literature report on the antibacterial efficacy of sulfonium moiety endorsed us to investigate the antibacterial activities of the **3.SL1a** lipid.<sup>15</sup> The antibacterial activity of the **3.SL1a** lipid was investigated against both Gram-negative (*E. coli*; MTCC-1687) and Gram-positive (*S. aureus*; MTCC-96) bacteria. The minimum inhibitory concentration (MIC) values were  $32 \pm$

2 and  $14 \pm 1 \mu\text{M}$ , respectively. However, the MIC values were  $18.75 \pm 4.0$  and  $10.00 \pm 3.0 \mu\text{M}$  for *E. coli* and *S. aureus*, respectively, in the presence of Dox@**3.SL1a**. The morphological analysis of bacterial cells in the presence and absence of Dox@**3.SL1a** through FESEM imaging showed a noticeable loss of cell shape and integrity (Figure 3.3B, C). Overall, the antimicrobial studies revealed that this sulfonium-based amphiphile exhibits efficient antibacterial activities. Recent studies also revealed that the peptidoglycan of the bacterial cell wall promotes the invasiveness of breast cancer cells by upregulating the Toll-like receptor 2 expressions.<sup>16</sup> Therefore, the delivery carrier having both the function stimuli-responsive anticancer drug delivery efficacy with inherent antibacterial activity represents **3.SL1a**, a highly efficient anticancer delivery system.

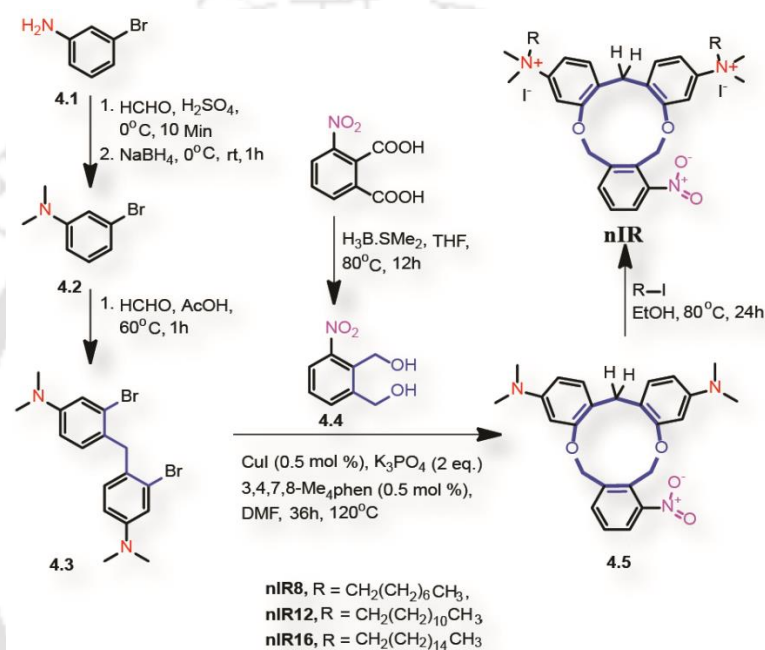


**Figure 3.3** Cartoon diagram representing the action **3.SL1a** liposome (A). FESEM images of *E. coli* (MTCC-1687S) treated with Dox@**3.SL1a** (B) and only Dox (C).

#### Chapter 4

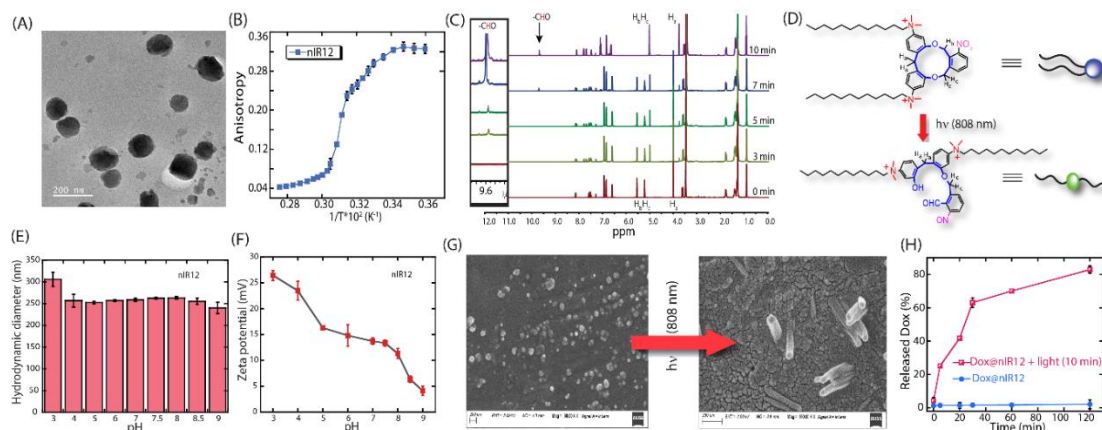
**Photoresponsive transformation from spherical to nanotubular assemblies: anticancer drug delivery using macrocyclic cationic gemini amphiphiles**

Although the scientific fraternity gives their best effort to design the novel endogenous stimuli-responsive delivery vehicle, we need smart externally controllable drug release tool to fight against cancer to precise drug delivery at the right time, right doses.<sup>1, 17</sup> In this regard, light controllable delivery vehicle are most promising one till date.<sup>18-20</sup> Here, we developed NIR-light-responsive macrocyclic cationic gemini amphiphiles, one of which displayed various favorable properties of lipids.



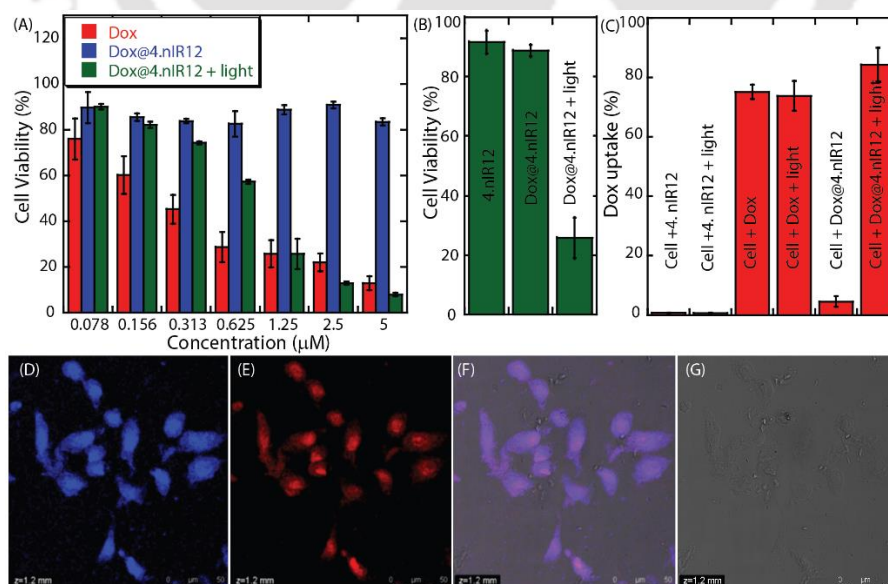
**Scheme 4.1** Synthetic route of the NIR-light active lipids.

The NIR-light-mediated cleavage of the strained dioxacycloundecine ring led to the conversion of the spherical to nanotubular self-assembly in the aqueous medium. The strained cyclic moiety of **4.nIR12** is linked with a photon absorbing 2-nitrobenzyl moiety. The 2-nitrobenzyl group depletes the energy by opening the constrained ring to an open-chain conformation on NIR-light absorption. Within the macrocyclic system, the molecular angular strain leads to drift from the tetrahedral geometry, which is liable to easy cleavage of the C–O bond via the Norrish type II mechanism.<sup>21</sup>



**Figure 4.1** TEM image of **4.nIR12** in the aqueous medium (A). Phase transition temperature (B)  $^1\text{H}$  NMR spectra of **4.nIR12** without and with NIR-light treatment at different time intervals (C). The pH-dependent hydrodynamic diameter (E) and surface potential (F) of NAs. FESEM images of the water-soluble aggregates of **4.nIR12** before and after NIR-light treatment (10 min) (G). Dox release profile from NAs of **4.nIR12** with and without NIR light irradiation (H).

This photo-mediated transformation from the spherical to nanotubular self-assembly resulted in the release of encapsulated hydrophobic anticancer drug molecule Dox in a controlled manner. The potent cationic gemini amphiphile also displayed lower cytotoxicity and efficient NIR-light-mediated Dox release efficacy to cancerous cells.

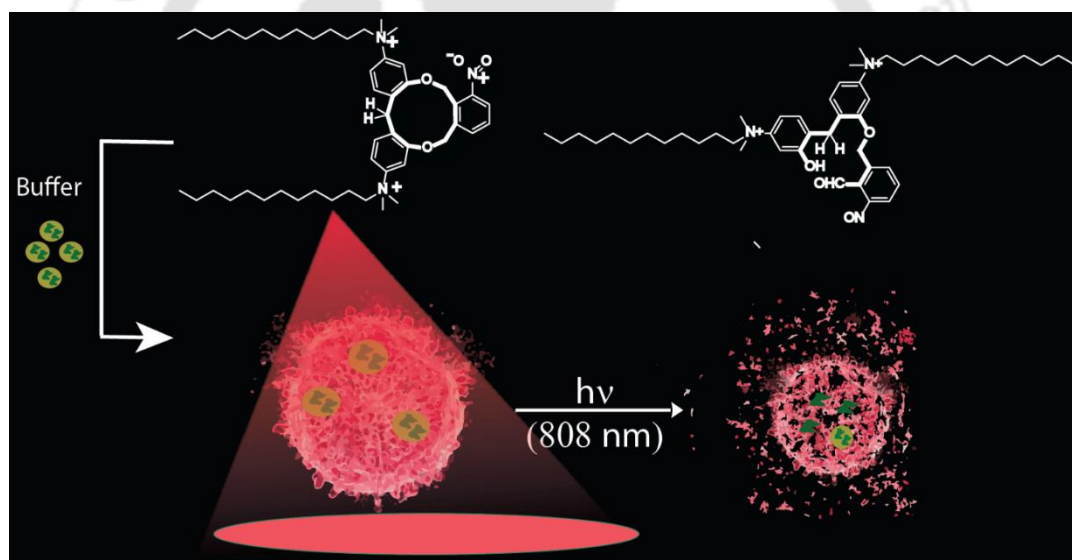


**Figure 4.2** Viabilities of MDA-MB-231 cells in the presence of free Dox and Dox@**4.nIR12** after 48 h of incubation (A). Viabilities of MDA-MB-231 cells in the



presence of **4.nIR12** (125  $\mu\text{M}$ ) and Dox@**4.nIR12** (1.25  $\mu\text{M}$  Dox and 125  $\mu\text{M}$  amphiphile) (B). Flow cytometry analysis to measure the Dox uptake efficiencies of MDA-MB-231 cells treated with **4.nIR12**, free Dox (125  $\mu\text{M}$ ), and Dox@**4.nIR12** (1.25  $\mu\text{M}$  Dox and 125  $\mu\text{M}$  **4.nIR12**) (C). Representative CLSM images of the MDA-MB-231 cells were incubated with Dox@**4.nIR12** (1.25  $\mu\text{M}$  Dox and 125  $\mu\text{M}$  amphiphile) for 8 h, followed by NIR-light treatment for 10 min.

Hence, this simple and innovative concept of developing macrocyclic gemini cationic amphiphiles as potential DDSs could be useful in photodynamic therapy, which could be dissected in a controlled fashion using NIR-light to liberate the enveloped hydrophobic chemotherapeutic drug molecules in a spatially- and temporally-controlled manner for surface and deep-tissue cancer treatment.



**Figure 4.2** Graphical representation of NIR-light active lipid and its anticancer drug delivery activities.

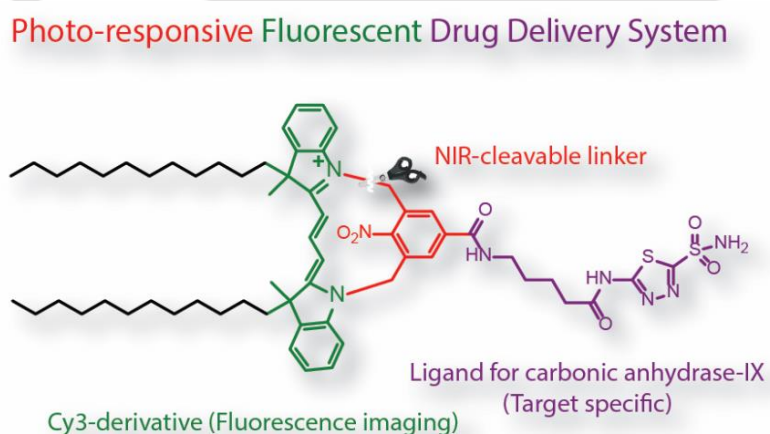
## Chapter 5

### Development of photoresponsive fluorescent lipid with cancer cell targetable ligand

In my previous chapter, we report a photoresponsive anticancer drug delivery system. However, early diagnosis or image-guided chemotherapy is also challenging due to the lack of suitable early diagnostic technology and the severe side effects associated

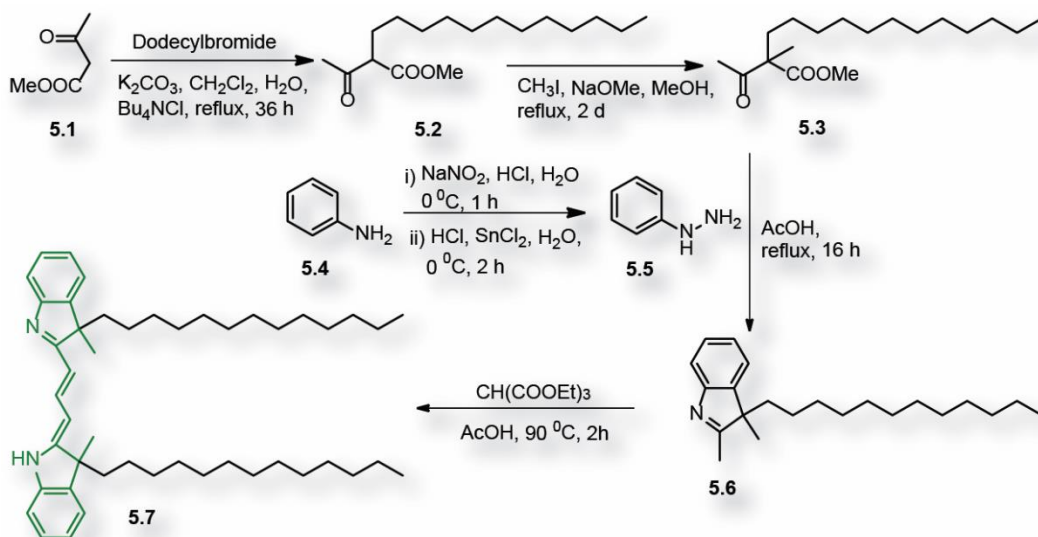
with the nontargeted fluorophore resulting from a lack of specificity for cancer cells.<sup>22, 23</sup> Thus, the development of targeted imaging agents is highly beneficial for diagnosis.<sup>24</sup> The NIR-light-responsiveness could provide on-demand delivery of appropriate drug doses in a spatially- and temporally-controlled manner for surface and deep-tissue cancer treatment. Therefore, developing multifunctional DDSs with fluorescence properties, targetability, and stimuli-responsive spatiotemporally controllable drug-release profiles is of great interest for chemotherapy.

Herein we developed acetazolamide-grafted cyanine-3 (Cy3) dye-based NIR-responsive macrocyclic amphiphiles. The Cy3 dye is useful for both fluorescence, and scattering-based imaging.<sup>25, 26</sup> The acetazolamide moiety present on the outer surface of the liposome could bind to the tumor-specific CA-IX receptor and deliver the chemotherapeutic drug molecules to the cancer cells.<sup>27</sup> The presence of a strained 12-membered cyclic ring and a photon absorbing *o*-nitrobenzyl moiety would make the amphiphile NIR-light-responsive.<sup>28</sup> In addition, the presence of Cy3 dye in the core group of the amphiphile could be useful for monitoring the cellular uptake and localization of the drug delivery systems.

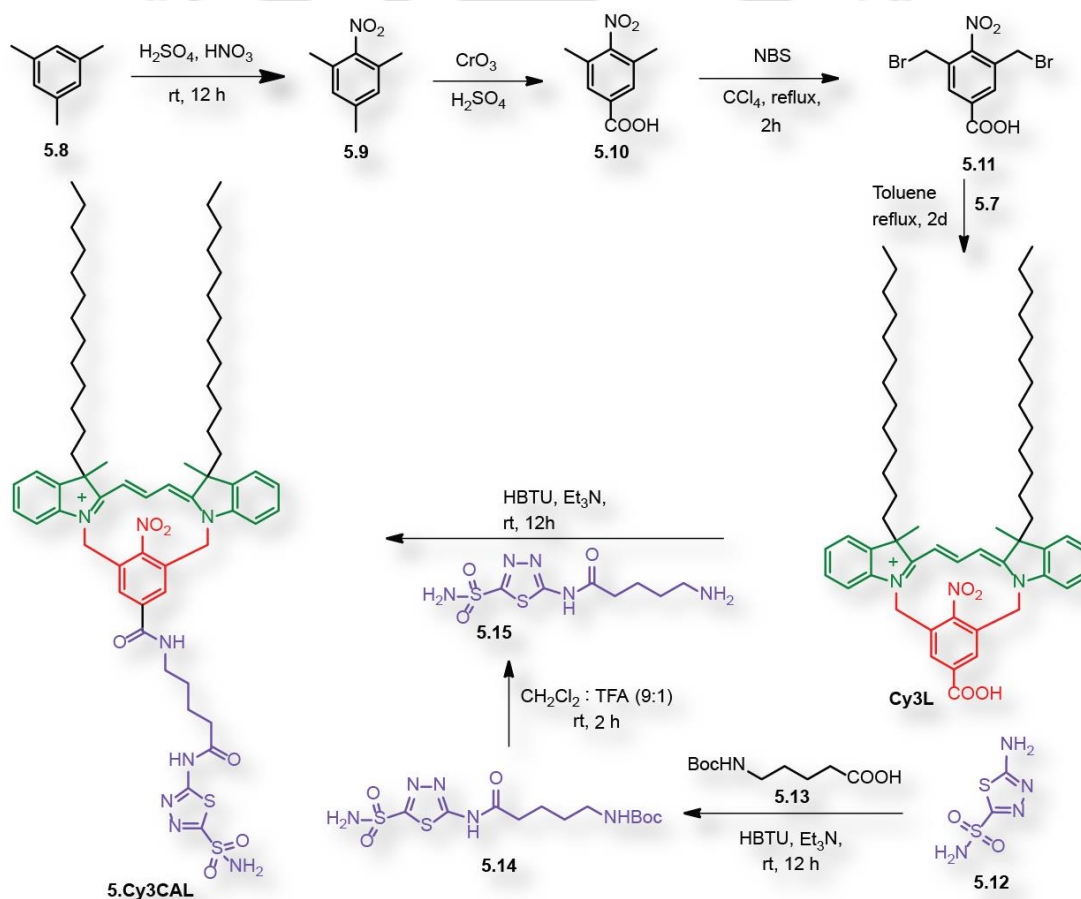


**Figure 5.1** Designed lipid molecule for photoresponsive image-guided targeted delivery.

We synthesize the lipid molecule from a few primary precursors modified cyanine3 dye, photo-active linker, and CA-IX enzyme targetable ligand acetazolamide derivative are synthesized separately.



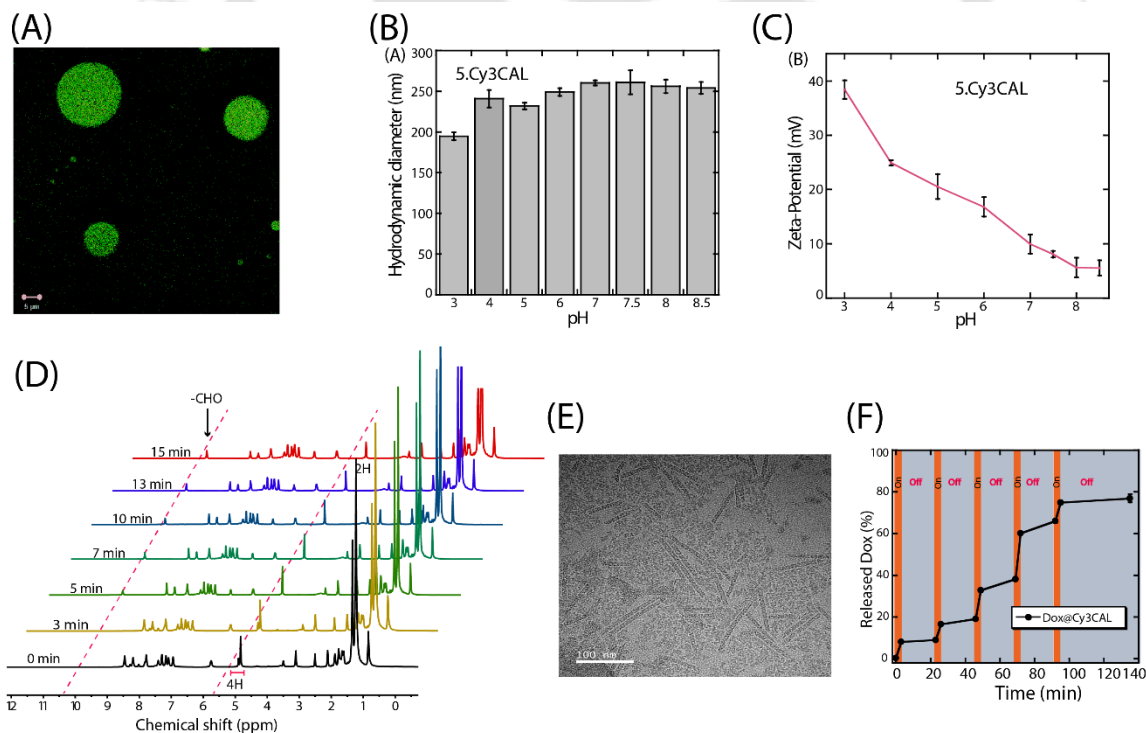
**Scheme 5.1** Synthesis of cyanine-3 derivative.



**Scheme 5.2** Synthesis of photo linker (5.11), acetazolamide derivative (5.15), and final lipid molecule (5.Cy3CAL).

Due to the amphiphilic nature, the **5.Cy3CAL** is anticipated to form soluble aggregates in the aqueous medium. The confocal laser scanning microscopic (CLSM)

analysis of the giant unilamellar vesicles (GUVs) of only **5.Cy3CAL** demonstrates its spherical assembly in PBS buffer, pH 7.2 (Figure 5.2A), which could be due to the formation of cyclic coulomb-coupled J-aggregates.<sup>29</sup> The analysis of transmission electron microscopic (TEM) and field emission scanning electron microscopic (FESEM) images also revealed the formation of spherical nano-sized aggregates (NAs) with diameters of 100–300 nm. The dynamic light scattering (DLS) analysis showed that the hydrodynamic diameter ( $d_H$ ) of the vesicles varies from 225–275 nm across different pH (Figure 5.2B), which signifies that the pH of the solution has a negligible effect on the hydrodynamic diameter of the vesicles. The surface potential decreases with the increase in pH (Figure 5.2C), which minimizes the positive charge of **5.Cy3CAL** by the increasing solvation number of the negatively charged hydroxyl group. The pH-dependent surface potential of amphiphile **Cy3L** showed -5.0 mV at physiological pH due to the presence of the acid group, which acts as a head group of NAs.

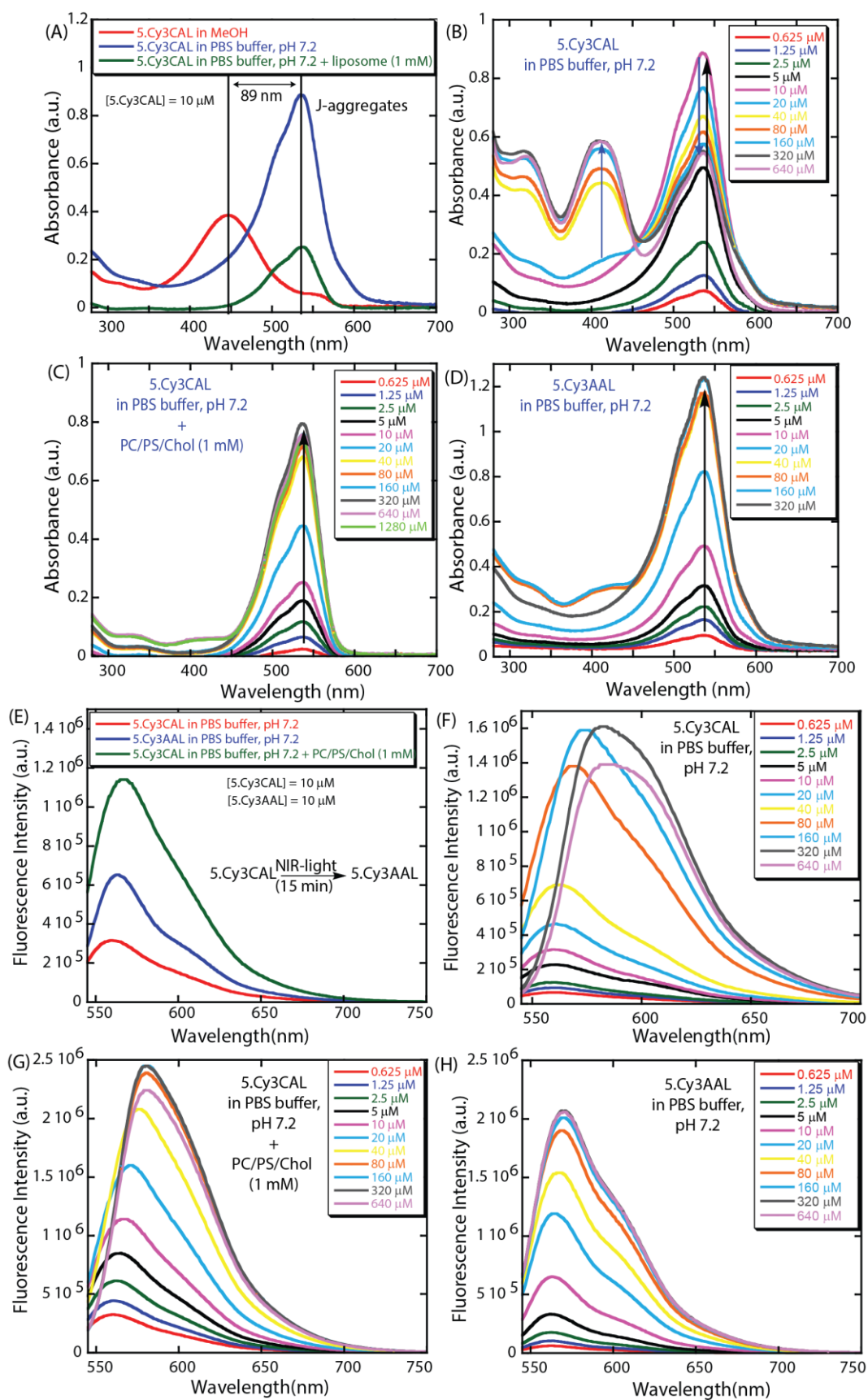


**Figure 5.2** GUV of **5.Cy3CAL** (A), pH-dependent hydrodynamic diameter (B) and zeta potential (C).  $^1\text{H}$  NMR spectra of **5.Cy3CAL** without and with NIR-light treatment at different time intervals (D). TEM images of the water-soluble aggregates of **5.Cy3CAL** before and after NIR-light treatment (15 min) (E). Dox release profile from NAs of **5.Cy3CAL** during batch-wise NIR light irradiation (F).

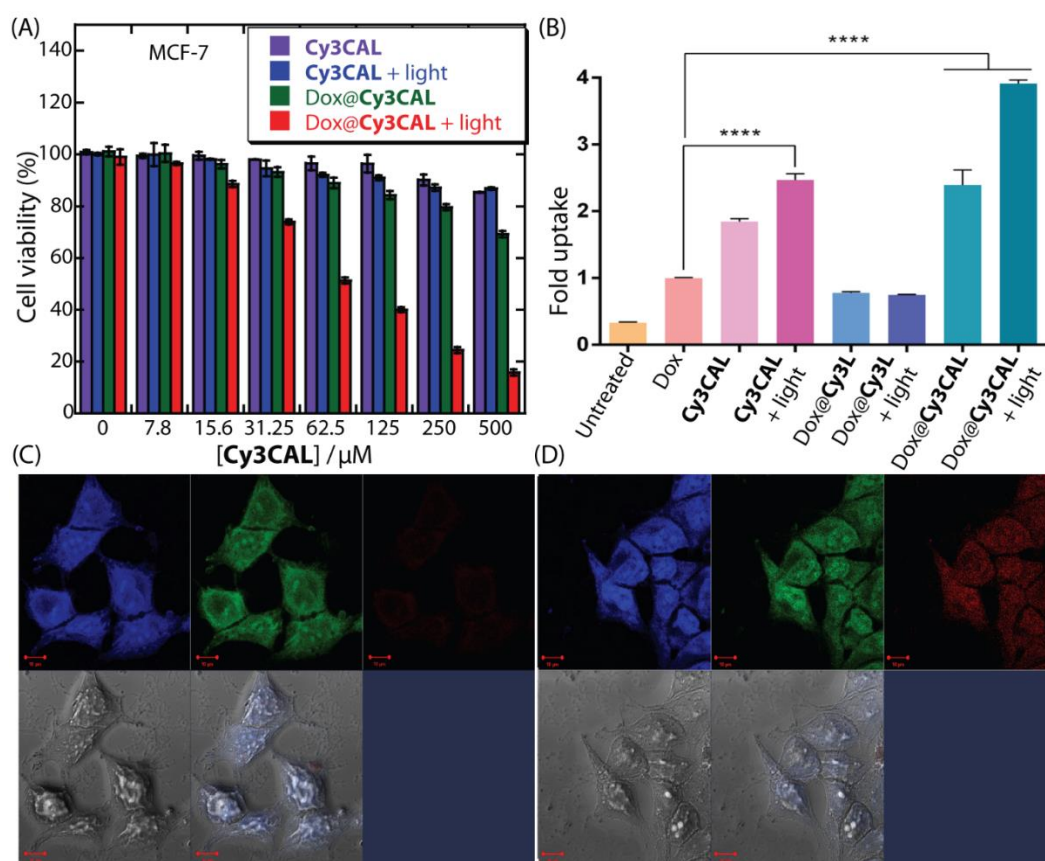
Fluorescence anisotropy measurements were performed to investigate the thermal stability of NAs of **5.Cy3CAL** in the aqueous media. The higher phase transition temperature ( $T_m = 53\text{ }^{\circ}\text{C}$ ) of the amphiphile suggests the higher thermal stability of the vesicles, which could be due to the formation of stable J-aggregates. The higher  $T_m$  values of only **5.Cy3CAL** demonstrate its higher thermal stability and drug retention capability, which could be useful in drug delivery and others.

The photo-induced transformation of macrocycle to acyclic amphiphile was investigated by  $^1\text{H}$  NMR analyses. A solution of **5.Cy3CAL** in  $\text{DMSO-}d_6$  solvent was irradiated with NIR laser light ( $808\text{ nm}$ ,  $1\text{ W cm}^{-2}$ ) for 0-15 minutes. The time-dependent  $^1\text{H}$  NMR analyses displayed the disappearance of one of the benzylic protons  $\text{H}_a$  ( $\delta = 3.8\text{ ppm}$ ) after 15 minutes of light irradiation and simultaneous generation of aldehydic proton at  $\delta = 9.8\text{ ppm}$  (Figure 5.2D). The disappearance of the proton signal of  $\text{H}_a$  indicates the cleavage of the *o*-nitrobenzyl moiety and the opening of the 12-membered strained macrocyclic ring. Hence, the presence of a photon-absorbing 2-nitrobenzyl group makes **5.Cy3CAL** amphiphile a NIR-sensitive molecule leading to the cleavage of the benzyl bond via the Norrish type II mechanism. Meanwhile, the absorption spectra of **5.Cy3AAL** also displayed a bathochromic shift in the aqueous medium ( $\lambda_{\text{ex}} = 538\text{ nm}$ ; in PBS buffer pH 7.2) compared to that in methanol medium (monomer;  $\lambda_{\text{ex}} = 446\text{ nm}$ ), suggesting the formation of fluorescent J-aggregates in the aqueous medium (Figure 5.3A). The fluorescence spectral analysis ( $\lambda_{\text{ex}} = 538\text{ nm}$  and  $\lambda_{\text{em}} = 565\text{ nm}$ ) revealed that the CAC values of **5.Cy3AAL** are around 25.28 (CAC1) and 89.44 (CAC2)  $\mu\text{M}$  in PBS buffer pH 7.2 (Figure 5.3F). The aggregation pattern of the NAs of **5.Cy3CAL** amphiphile after light irradiation in the aqueous environment was also analyzed by the TEM and FESEM analyses. The images revealed that the spherical NAs turn into rod-like nano assembly after NIR-light treatment (Figure 5.2E). The result indicates that after NIR light irradiation, the unsymmetrical (headgroup) **5.Cy3AAL** molecules could form oblique-type J-aggregation patterns to form nanotubes.<sup>30</sup> The formation of such fluorescent nanotubes could be useful for light-harvesting applications.<sup>31, 32</sup> We hypothesized that this photo-induced conformational change could release the encapsulated drug molecules for the NAs of **5.Cy3CAL** amphiphile.





**Figure 5.3** UV and fluorescence analysis of 5.Cy3CAL before and after light irradiation also in the presence and absence of natural lipids.



**Figure 5.4** Viability of cell MCF-7 (A). FACS analysis to investigate the uptake by MCF-7 cells (B). Representative CLSM images of the MCF-7 cells treated with Dox@5.Cy3CAL before (C) and after (D) NIR-light treatments.

The synthesized Cy-3-based fluorescent amphiphile exhibits a visible J-aggregation bathochromic shift in the aqueous and targeted drug delivery efficacy to the cancer cells. Photo-induced irreversible transformation spherical to nanotubular self-assembly and the presence of cancer cell recognizing ligand is appealing for various applications, including targeted drug delivery and early diagnosis or image-guided chemotherapy.

## Conclusion

This thesis has successfully described the role of a drug delivery system (DDS) and its progress toward developing smart delivery vehicles in **Chapter 1**. The proper functionalization and a milder approach for the synthesis of cationic lipid (in **Chapter 2**) show the DNA delivery efficacy. To overcome the limitation of the conventional DDS, sulfonium moiety-based GSH-responsive anticancer DDS (in **Chapter 3**) was developed. The potent lipid has shown the cancer cell-specific Dox



deliver with inherent antibacterial activity over gram-positive and gram-negative bacteria. A photoresponsive amphiphile (**Chapter 4**) describes how exogenous stimuli can control the drug release profile and targeted delivery without relying on endogenous stimuli. Development of multifunctional photoresponsive image-guided targeted anticancer drug delivery (in **Chapter 4**) emphasizes the appositely delivery and imaging of preclinical and clinical applications of theranostic nanoplateforms.

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### Future Prospects

Over the last few decades, significant advancement has been made in liposome-based therapeutics delivery. It has been getting more attention because it enhances the therapeutic potential of several cytotoxic drugs (like Dox). So motivated by these therapeutic prospects, a diverse range of novel lipid molecules has been developed throughout this thesis. However, the selective delivery between a normal cell versus a cancerous cell is a substantial hurdle to date. In addition, the heterogeneity and metamorphism behavior of cancer cells make an immense challenge to cancer cell-targeted drug delivery and demand better understanding to develop a smart carrier. The use of passive or active multi-targeted drug delivery systems could be a practical approach for therapeutic purposes. The development of smart multi-functional vehicles can be explored in the future.

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## *Chapter 1*

### *Introduction of liposome-based DNA/drug delivery*

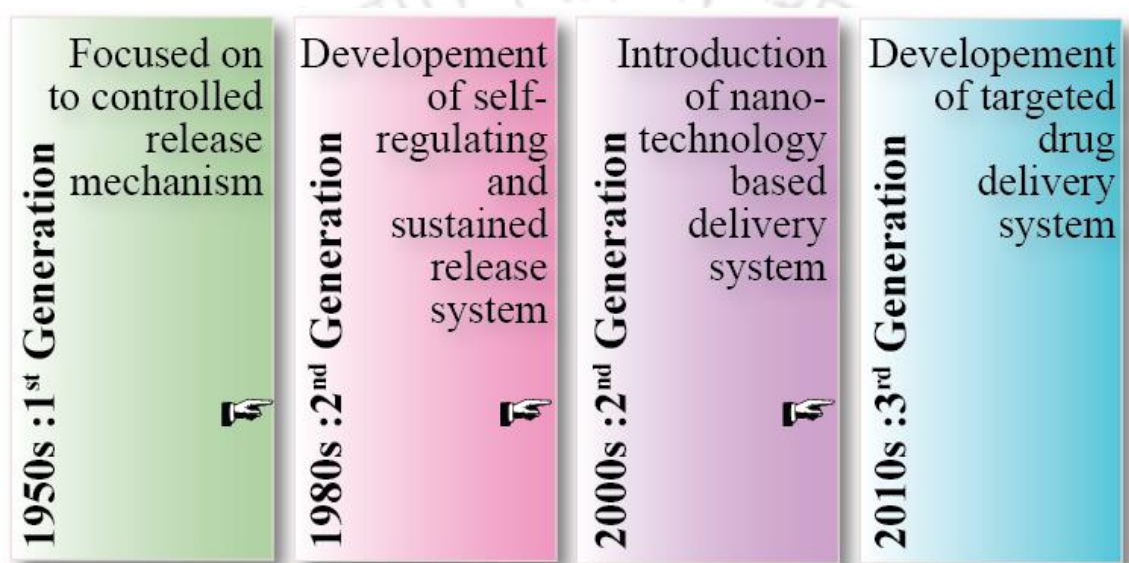




## 1.1. Introduction

Before the 1950s, all drugs were made into pill or capsule formulations that released the loaded drug straightway upon contact with water without any ability to control the drug release kinetics from the capsule. In 1952, Smith Klein Beecham introduced the first sustained release formulation that was able to control the drug release kinetics at a predetermined rate with a half-life of 12-hour and the technology known as Spansule technology.<sup>1</sup> In the very next year, the Nobel Prize in chemistry was awarded to Professor Hermann Staudinger for demonstrating the existence of "Makromoleküle" macromolecules; later, the concept of macromolecule was emphasised in the field of sustained drug release formulation, and in 1955, Jatzkewitz reported the preparation of the first polymer-drug conjugate, polyvinylpyrrolidone-mescaline conjugate, which contained a dipeptide (glycyrrhizin) spacer between the drug molecule and the polymer and can be considered as the first nanoparticle (NP) therapeutic.<sup>2-3</sup> Later on, lipid vesicles were discovered by Alec D. Bangham and described the potential as a drug delivery system (DDS) for the controlled release with cell high permeability.<sup>4</sup> Polymer-drug conjugates and liposomes mark the birth of nanocarriers based drug delivery, and together these two domains of nanocarriers represent most of the marketed nanomedicine and continue to be investigated extensively.<sup>3</sup> Early stages of drug delivery, most efforts are dedicated to the fundamental understanding of the drug release mechanisms controlling physicochemical properties, such as water solubility and cell permeability, which were adequate enough to establish *in vitro-in vivo* correlation (IVIVC).<sup>5</sup> Most of the drug delivery systems developed in the period, mainly for oral and transdermal delivery, understanding the physicochemical properties (e.g., *in vitro* drug release kinetics) was enough for developing clinically useful formulations. However, no biological barriers were identified for those formulations. The drug delivery systems developed during the second generation (1980 - 1990) dealt with more complex problems to the biological barrier. Therapeutic release formulations were designed to achieve zero-order release kinetics, but the release achieved in various *in vitro* dissolution systems did not reflect in the maintenance of the constant drug concentration *in vivo* system. This period also introduced various sustained-release formulations of peptide/protein-drug conjugate after grafting in the body.<sup>1, 6</sup> The drug release profile from a

formulation *in vivo* depends on the formulation types and the formulation types biological obstacles like the environment surrounding the implanted formulation. At the beginning of the 21st century, the ongoing "nanomedicine euphoria" started because nanosized materials present characteristics that differ from those of larger materials and can deliver therapeutics efficiently. This led to the application of nanotechnology in medicine (nanomedicine) for various aspects of diagnostic, therapeutic, and prognostic purposes and was later extended for the improved treatment of cancerous tissues.



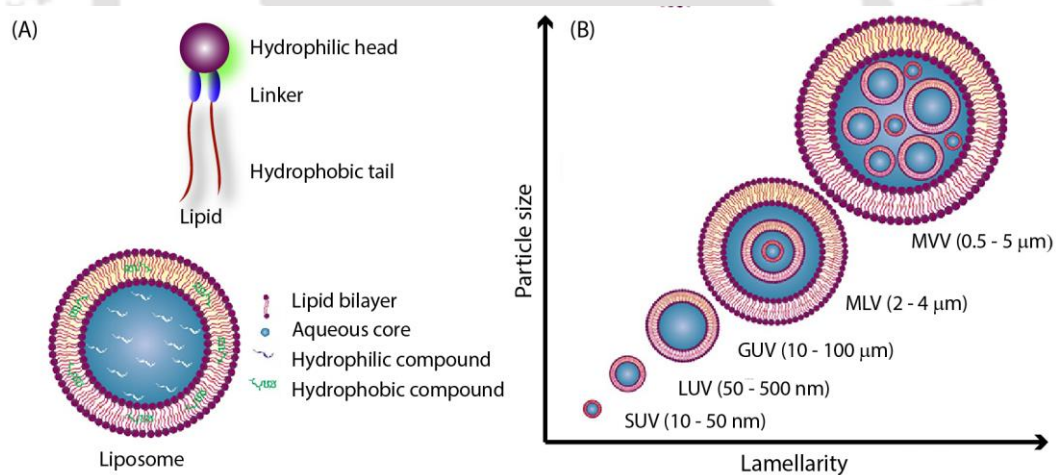
**Figure 1.1** Generation-wise development of drug delivery system.

The continuous efforts are dedicated to a suitable drug delivery system to ensure a higher and longer duration of drug bioavailability and thereby enhanced therapeutic efficacy. Several materials with different structural properties are constantly evolving toward smart delivery vehicles. The most commonly used drug delivery vehicles are liposomes, viral vectors, self-assemble proteins, metallic nanoparticles, different types of polymeric nanoparticles, micelles, etc.<sup>7-11</sup> The ultimate goal of this technology is to achieve a robust, targeted delivery system that can load a sufficient amount of multiple therapeutic and diagnostic agents for apposite release to the targeted site with no adverse side effects.

## 1.2. Liposome as a DNA/drug delivery vehicle

Liposome was first described by Alec D. Bangham (British hematologist) in 1961.<sup>12</sup> Liposomes are self-assembling closed colloidal structures composed of lipid bilayers.

The polar head group interacts with water molecules, while the hydrophobic tails escape from water molecules and interact with each other, and have a spherical shape in which an outer lipid bilayer surrounds a central aqueous space. The bilayer can form in different vesicle sizes (diameter about 15-3500 nm) and shapes (single and fused particles). Initially, it was used to construct a model membranes system for biological study. The possibility of using liposomes vesicle as a therapeutic loading and carrier agent was introduced in 1968 by Bangham, even though it took many years before the appropriate technologies were in place.<sup>12</sup> Later on, Liposomes have gained more attention from being just another exotic object of biophysical research to becoming a pharmaceutical carrier. The real breakthrough improvements as a delivery vehicle over the past 30 years have resulted in the approval of several liposomal drugs and many under clinical trials.<sup>13-14</sup> Liposomes themselves range from multilamellar vesicles (MLVs) with diameters of several microns to small, unilamellar vesicles (SUVs) with diameters of typically 15-30 nm, while large unilamellar vesicles (LUV) range from 100-200 nm or larger.<sup>15</sup>

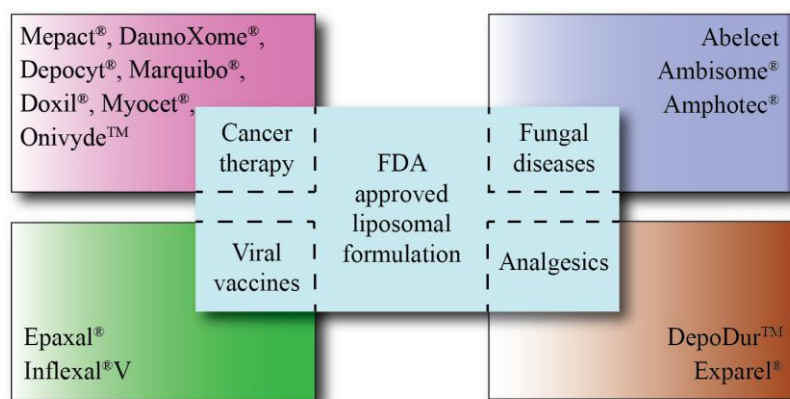


**Figure 1.2.** Schematic representation of lipids, liposomes, and different types of vesicles.

However, LUV has become the greatest utility in biomedical delivery applications, having diameters centered around 200 nm, which is large enough to carry a significant payload and small enough to slip between leaky endothelial junctions, and these are now more commonly known as lipid nanoparticles (LNs). For instance, due to the specific structure of liposomes having hydrophilic polar and hydrophobic nonpolar cores, both groups of hydrophilic and hydrophobic drugs can be entrapped in them easily. The encapsulated components within this lipid bilayer are protected

from extra-liposomal reactions that could alter the efficacy of the therapeutic molecules by enzymatic degradation or modification of it.<sup>16</sup> Moreover, LNs could enhance the therapeutics accumulation at desired sites of action and reduce the toxic effect in the healthy tissues. LNs offer other several advantages, including the system can be tuned with different composition or functionality for the constant zero-order release rate (as diffusion out of a membrane is concentration dependent, and the release would depend on the permeability profile of the encapsulated drug) to maintain a constant therapeutic dose at the site of action, reduced clearance to increase the drug half-life, biocompatibility, non-immunogenic, ability to carry large drug payloads, and a wide range of physicochemical and biophysical properties that can be modified to control their biological characteristics. Usually, a delivery vehicle functions by either passive pathways or active targeting pathways.<sup>17</sup> In passive targeting, the LNs have been developed in accordance with physicochemical properties of the diseased tissues as observed with many formulations of conventional anticancer drugs. Cancer cells possess vascular tissue that is porous and rather leaky, and the endothelial cells often have large gaps between them that allow LNs with diameters up to 400nm to extravasate from the blood into the surrounding tissues, where they concentrate.<sup>18</sup> Also, in the passive pathway long circulation period will allow more accumulation of the nanoparticles to the tumor site through the enhanced permeability and retention or enhanced permeability and retention (EPR) effect.<sup>21</sup> EPR was referred to as a universal pathophysiological phenomenon and mechanism in which macromolecular compounds such as nanoparticles, LNs loaded drugs, albumin, etc. beyond certain sizes ( $\geq 40$  kDa) can gradually accumulate in the tumor vascularized sites compared to normal tissues or organs and achieve maximum retention of anticancer drug in cancer tissues after a certain period. However, if the lipid nanoparticles attach to vascular endothelial cells via a non-internalizing epitope, high drug concentration will be available on the target cells' microenvironment. The internalization of LNs based drug delivery systems has shown an enhanced therapeutic effect.<sup>22-25</sup>





**Figure 1.3.** Few FDA-approved liposome-based drug formulations.

In active targeting, the surface of LNs is modified with targeting moieties (e.g., ligand, antibody, peptide) to target specific changes in disease cell biology, which are highly upregulated in comparison to the healthy surrounding cells and tissues. The targeting moieties play an important role in cellular uptakes, such as RGD-peptides have a relatively high and specific affinity for  $\alpha\beta_3$  integrins that over-express in tumor neovasculature.<sup>19</sup> Conjugation of the RGD-peptide important technology has been widely used for the early diagnosis and differential diagnosis of tumors and clinical analysis and treatment. Ligand acetazolamide derivatives were known for decades to bind or inhibit carbonic anhydrase (IX) or CA-IX, which is more prevalent in solid tumors. As a result, CA-IX has been considered a therapeutic target for aggressive cancers.<sup>20</sup> Recent advances in technology have demonstrated that lipid nanoparticles (LNs) represent one of the most promising colloidal delivery systems that efficiently encapsulate and deliver therapeutic agents ranging from small, conventional drug molecules to plasmid DNA containing therapeutic genes. During the last decade of the twentieth century and onwards, novel lipids, namely polyethylene glycol [PEG]-conjugated lipids and strongly cationic lipids<sup>26</sup>, emerged for genetic material carriers and allowed the design of LNs capable of efficient encapsulation of DNA- and RNA-based drugs.<sup>27-28</sup>

### 1.3. Synthetic liposomal nanoparticles for DNA delivery

Nowadays, gene therapy technology is a well-recognized approach in treating genetic hereditary diseases and holds potential for cancer treatment, wound healing, genetic vaccination, immunomodulation, and AIDS.<sup>29-30</sup> It provides a unique approach to treating both inherited and acquired diseases by delivering genetic

material to the targeted tissues protectively to replace the mutated gene or abnormal gene.<sup>31</sup> However, the real challenge for successfully executing this technology is the vectors used to deliver the transgene into host cells. Two different categories of vectors are mainly used for gene transfer: viral and non-viral gene delivery.<sup>32-33</sup> Viral vectors have been preferred for several clinical applications due to their efficient intracellular DNA delivery capability.<sup>8, 34</sup> However, the application is limited by the adverse effect of host immune and inflammatory reactions, the economic burden of production, the potential to form replication-competent virions, the size limit of the exogenous DNA, and the risks of inducing tumorigenic mutations and generating active viral particles through recombination.<sup>35-36</sup> These limitations of viral vectors have driven research to find a more effective and reliable system to deliver the gene.<sup>32, 37-38</sup> Among the existing arsenal of non-viral vectors, Cationic liposome-DNA complexes (lipoplexes) response potentially feasible and distinct advantages to viral vectors for the delivery of therapeutic genes. Cationic liposome provides the following advantageous properties: simplicity of preparation, good repeatability and biodegradability, potential commercial value, and a wide range of clinical applications and safety.<sup>39</sup> A huge number of positively charged head group lipid has been developed to make the complex of positively charged with negatively charged phosphate group in nucleic acids and form uniquely compacted structure called lipoplexes.<sup>40-41</sup> Gene transfection efficacy depends on overall geometric shape, charge density per molecule, and cellular uptake feasibility. Lipoplexes are easily recognized by the cell membrane due to their positive surface charge electrostatically interacting with negatively charged proteoglycans and glycoproteins of the cell membrane, which may induce the cellular uptake of nucleic acids. Also, these positively charged lipids protect the genetic material from the intracellular and extracellular nuclease. In contrast, using a cationic polyamine head group containing amphiphile reduces the half-life of lipoplexes during blood circulation, limiting its utility beyond vascular endothelial cells.<sup>41a</sup> The circulation half-life and lipoplex stability can be significantly enhanced by using neutral polymers like polyethylene glycol (PEG) in the composition. The lipid composition can be varied and can include additional components, such as stabilizer, Fusogenic lipid (DOPE) with cationic lipid, usually *N*, *N*-dioleoyl-*N*, *N*-dimethylammonium chloride (DODAC), and PEG-ceramide (PEGCer).<sup>42-43</sup> However, developing a non-



viral vector satisfying the several parameters demands a more deep understanding and designing of proper lipid molecules, and it should be easy to scale up. Also, natural lipid nanoparticles have some disadvantages because of their perfect crystalline structure and low drug loading efficiency and stability.

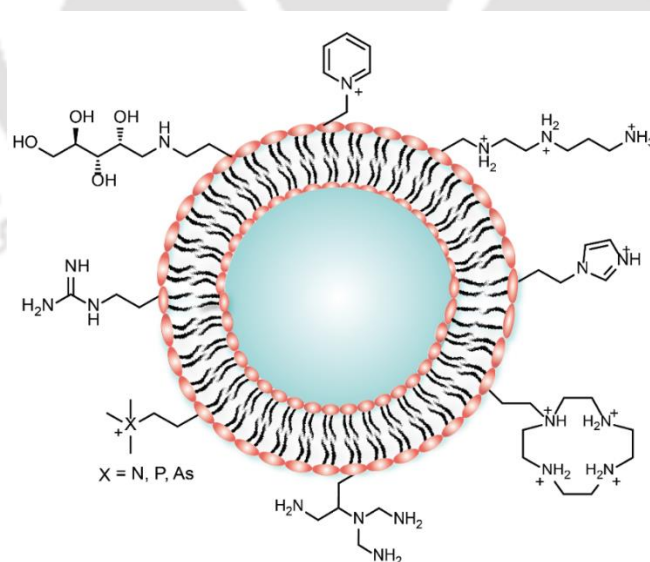
#### 1.4. Structural evolution of the head, linker, and tail group of cationic lipid for DNA delivery

Cationic lipids used as carriers for genetic material delivery are generally composed of a three central domain a cationic polar head, a hydrophobic lipid unit, and a spacer linker group that connect the first two moieties. In the field of cationic lipid as a non-viral vector for gene delivery, the pioneering studies of Felgner and Behr are notable. Indeed, Felgner first reported that liposomes could form compact complexes with pDNA called lipoplexes and protect pDNA as well as transfect cells.<sup>44</sup> In this work showed the capability of delivering plasmid DNA into living cells using cationic liposomes with unnatural glycerol backbone-based cationic transfection lipid-DOTMA. Since this early work, a wide variety of synthetic lipids have been reported for gene delivery with extensive modification in the head, linker, and hydrophobic tail groups.<sup>45-49</sup> At the initial development phase of cationic lipid (1,2-bis(palmitoyloxy)-3-propyl dimethyl( $\beta$ -(p-nitrophenyl carbonato) ethyl)ammonium bromide, which mainly consists of two saturated aliphatic chains (palmitic acid, oleic acid), ester or amide linker, and quaternary ammonium group as a polar head group.<sup>50</sup> However, lipids are modified with different linkers, such as the more stable ether linkages (e.g., DOTMA) and more degradable carbamate linkages (e.g., DC-Chol; (3 $\beta$ -[N-(N',N'-dimethylaminoethane) carbamoyl]-cholesterol hydrochloride)), and different hydrophobic region with more rigid steroid derivative along with aliphatic chain (e.g., DC-Chol).<sup>51-52</sup> Until now, few of these have been used in gene therapy clinical trials for the treatment of cancer and genetic disorders, and several other amphiphiles have become commercially available as transfection reagents (such as Lipofectamine 2000, LipoTaxi, Lipofectin, TransFast, etc.) for the transfection of cell lines in the laboratory.<sup>53-55</sup> Synthetic development of cationic lipids as a component for gene delivery vehicles has become a major research tool for transferring exogenous genetic material into host cells. Synthetic cationic lipid-based gene delivery requires more understanding of the development of

sophisticated virus-like systems, which can be viewed as "programmed supramolecular systems".

#### 1.4.1. Design of cationic head group of lipid

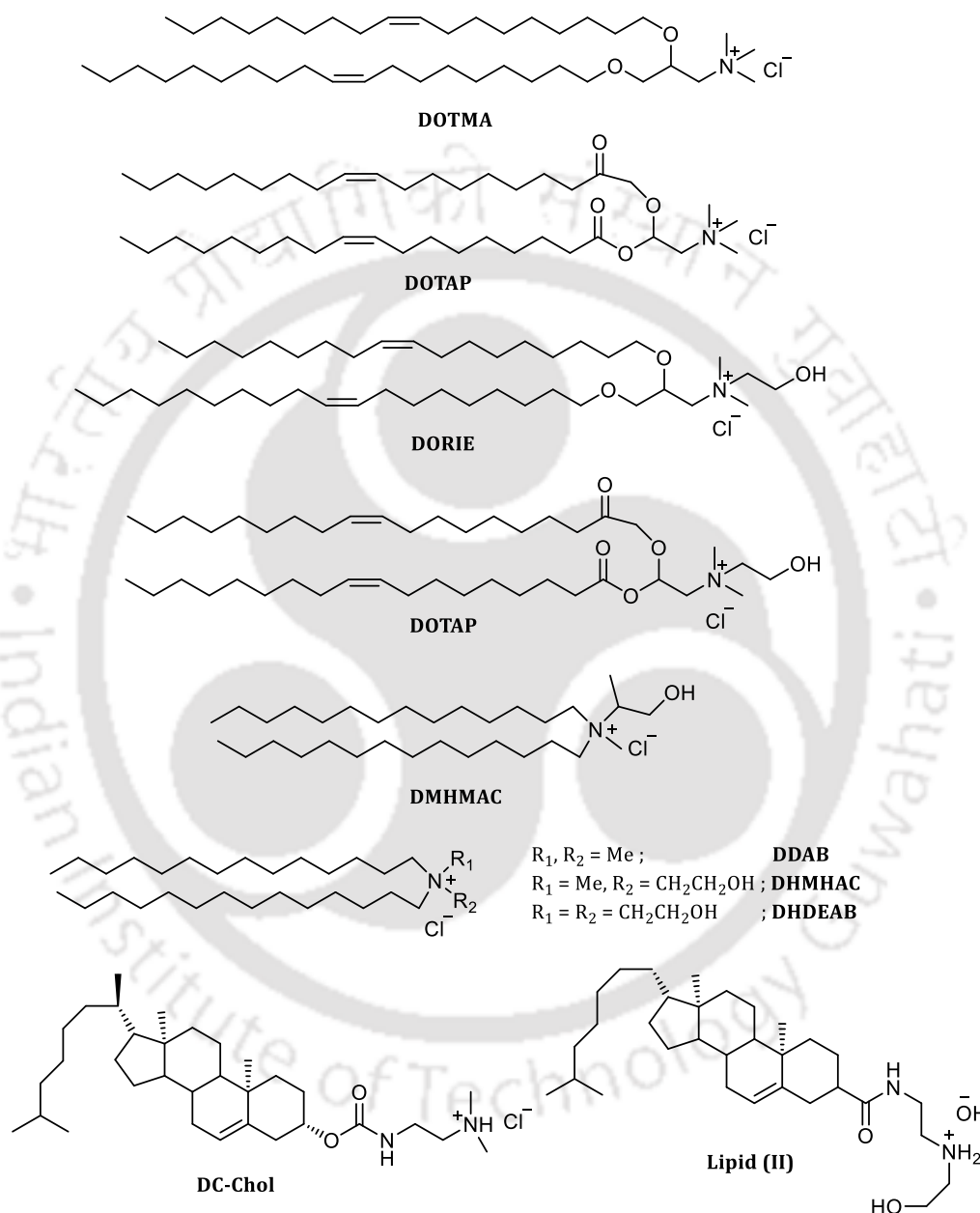
The vector's effective lipoplex formation requires a sustained positive charged headgroup at physiological pH. Generally, ammonium was broadly introduced as a charged head group at the early stages. However, other charged groups Phosphonium, arsonium, and metal ion coordinated groups, have since been employed. A comparison study showed that the phosphonium and arsonium vectors were less cytotoxic than the ammonium-based lipid.<sup>56</sup> However, the structure-activity relationship study revealed that mono-ammonium headgroups containing lipid have high hydration energy and high charge density to form compact lipoplex than other charged groups.<sup>56-57</sup> Lipoplex with higher fusion ability result in improved transfection efficiency.<sup>58</sup> Such lipoplex instability can be related to the formation of inverted hexagonal phases facilitating lipid mixing (especially in the presence of the hexagonal phase-forming co-lipid DOPE). Hydration of the cationic head group can be optimized by incorporating of hydroxyalkyl chain, which participates in hydrogen bonding with the imminent headgroup.



**Figure 1.4.** Liposome modification with different cationic head groups.

Thereby, transfection efficacy by the well-known lipids DOTMA (Figure 1.4) and the ester-linked variant DOTAP (1,2- dioleoyloxy-3-[trimethylammonio]-propane, (Figure 1.4) was improved by the introduction of a hydroxyethyl group to yield vectors DORIE (1,2-dioleoyl-3-dimethyl-hydroxyethylammonium bromide) and

DORI (1,2-dioleoyloxypropyl-3- dimethyl-hydroxyethyl ammonium chloride) respectively (Figure 1.4).<sup>57, 59</sup> However, in some cases, the high hydrating effect of DOTAP has been altered by attaching alkyl chains of increasing length instead of hydroxyalkyl group modifications in the aliphatic chain.<sup>60</sup>



**Figure 1.5.** Few reported cationic lipid molecules.

Takeuchi *et al.* reported another interesting agreement with a relationship between headgroup size and transfection efficiency that the dimethylamino headgroup of DC-Chol (Figure 1.5) showed higher gene transfection ability than its diethylamino and diisopropylamino derivatives.<sup>61</sup> Thus, the transfection ability of Lipid (II) (Figure

1.4) may be related to lipoplex instability found that monohydroxylated lipid DHMHAC (*N,N*-di-*n*-hexadecyl-*N*-methyl-*N*-(2-hydroxyethyl)ammonium chloride) and dihydroxylated lipid DHDEAB (*N,N*-di-*n*-hexadecyl-*N,N*-dihydroxyethylammonium bromide) of (Figure 1.5) were found to high levels of gene transfection ability than the DDAB (dioctadecyldimethylammonium bromide)).<sup>62-63</sup> The hydroxyethyl portion is present in a substituted form, such as in the vector DMHMAC (*N,N*-myristyl-*N*-(1-hydroxyprop-2-yl)-*N*-methylammonium chloride) (Figure 1.5). Lipid DMHMAC (*N,N*-myristyl-*N*-(1-hydroxyprop-2-yl)-*N*-methylammonium chloride) (Figure 1.5) was found to be twice as efficient as non-hydroxyl derivative DDAB/DOPE *in vitro* studies.<sup>64-65</sup> However, few other studies not confirmed the positive effect of the addition of a hydroxyl group into the cationic headgroup structure. Such as an amphiphile cholest-5-en-3 $\beta$ -oxyethane-*N,N*-dimethyl-*N*-2 hydroxyethylammonium bromide was found to be less efficient than its trimethyl non-hydroxyethylated homolog.<sup>66</sup> Polyamine-containing cationic lipids like spermine and polyethyleneimine are usually expected to be optimal interaction with DNA and delivery due to their greater surface potential than their monovalent counterparts. Notably, more protonation sites per molecule enhance the charge density, and lesser amounts of lipid show significant transfection activity.<sup>67</sup> Polymeric materials like poly-L-lysine as well as histidine histidine-rich polymers and peptides have been recognized as efficient gene delivery vectors.<sup>68</sup> Hence, multivalent cationic lipids exhibit high transgene expression with some degree of cytotoxicity.<sup>69</sup>

#### 1.4.2. Modification of the hydrophobic tail group

The choice of aliphatic chain or hydrophobic moieties in the synthetic cationic lipids significantly affects their transfection efficiency. Therefore, synthetic lipid vectors are often prepared in a library of different hydrophobic groups (aliphatic long chains, branched-chain, steroid domain, and fluorinated domain) to optimize the best combination. A detailed study of varying the saturated aliphatic chain length of C14, C16, and C18 over the different derivatives of DOTAP (alkyl acylcarnitine esters, bis-ether, glycine betaine derivatives) has shown that shorter chain length may facilitate intermembrane mixing, an essential factor in endosomal escape.<sup>39</sup> However, another study by Floch et al. showed that the transfection efficiency of a

series of phosphonolipids with varying alkyl chains of different lengths was found to be  $C14 > C_{oleyl} > C18 > C16 > C12$ .<sup>70</sup> Few reports demonstrate that aliphatic chains, single-tailed cationic lipids were originally reported to have low transfection efficiency and high toxicity in plasmid delivery compared to the cationic lipids with two, three and, four hydrocarbon chain.<sup>71</sup> However, Cameron et al. described a novel class of peptide-lipid conjugate with one palmitoyl chain as inferior gene transfection efficiency to the di- or trisubstituted counterparts.<sup>72</sup> Cationic lipids using cholesterol as a hydrophobic group can also improve the transfection efficiency because the cholesterol offers rigidity and a very stable bilayer structure and its endogenous biodegradability and fusion activity.

#### 1.4.3. Modification of the linker moiety

The linker region of the cationic lipids is extensively varied with different groups like amide, ester, ether, carbamate, etc. The linking bond gives the stability of the cationic amphiphile, and so a balanced choice must be made between the delivery vehicle stability and its toxicity which is likely to be related to its half-life in the cell. Linker moieties that are sensitive to stimuli such as the decrease in pH, change in redox potential, enzymes, and photon irradiation also plays a crucial role in stimuli-responsive delivery.

#### 1.5. Synthetic liposomal nanoparticles for anticancer drug delivery

Chemotherapy is a general approach to fight against cancer, one of the leading causes of death globally. However, the approach is not safe enough until the potent drug molecule is delivered to the cancer tissues selectively, as the anticancer drug showed some serious toxicity, even death. Till now, 5-fluorouracil (5-FU) has been widely used clinically for colon cancer, breast cancer, and gastrointestinal cancers; Dox is also used to treat different types of cancers (breast, bladder, kidneys, ovaries, thyroid, stomach, lungs, bones, etc.).<sup>73-75</sup> Unfortunately, drug molecules' toxic effects and fast inactivation have limited their further clinical use. Several drug delivery systems have been reported to minimize the side effects of these drug molecules and enhance therapeutic efficacy. In this regard, liposome-based anticancer drug delivery agents represent an advanced technology among the nanomedicine systems developed so far.<sup>76</sup> Myocet®, Doxil®, depocyt®, and other several liposomal drug



formulations were approved by the food and drug administration (FDA), which describes the potential of a liposome-based drug delivery system.<sup>13</sup> Moreover, along with safeguard of the therapeutics drugs, the liposome surface can be modified with various cancer cell targeting ligand as well as fluorescence counterparts for theranostics applicability. The design of lipid molecules with inherent diverse functionality offers a pivotal role in the successful deliverability of the therapeutics.

## **1.6. Stimuli-responsive drug delivery system**

The scientific fraternity is currently making a huge effort to develop nanoscale-based drug delivery systems to treat cancerous cells, such as integrated prodrugs, polymeric micelles, lipid-based drug delivery systems, and inorganic nanoparticles, nanotubes, and dendrimers.<sup>77</sup> The aforementioned drug delivery strategies have their promising characteristic features. Nevertheless, stimuli-responsive lipid-based drug delivery strategies have revolutionized the chemotherapeutic approaches in terms of enhanced bioavailability and selective cytotoxicity to the cancer cells compared to the conventional approach. The stimuli-based drug delivery system includes the control over drug release via various mechanisms and is known as "stimuli-responsive materials". Stimuli-responsive materials are those that undergo a physical or chemical, or structural change in response to a specific stimulus. The stimulus-responsive nanomedicines and nanotechnologies have a huge importance on target-specific delivery with a controlled drug release profile. These advanced carriers are developed with special chemical moieties (or constituent building blocks) that can respond to various stimuli. Typical stimuli could be applied internally within the tissue by internal triggers (pH, temperature, redox potential, etc.) or from external triggers (ultrasound, light, alternating magnetic field, etc.).<sup>78</sup> A vital role is also played by designing vehicles with a stimuli-responsive group for the spatiotemporally controllable release of the drug by the stimuli-inducing factors.

### **1.6.1. Internal Stimuli-Responsive drug delivery system**

#### **1.6.1.1. pH-Responsive delivery strategy**

Subtle pH variations between the pathological tissues, such as inflammation, infection, and cancer tissues from the normal tissues, could be used to control the release of chemotherapy agents for drug delivery. The pH at the tumor site is slightly

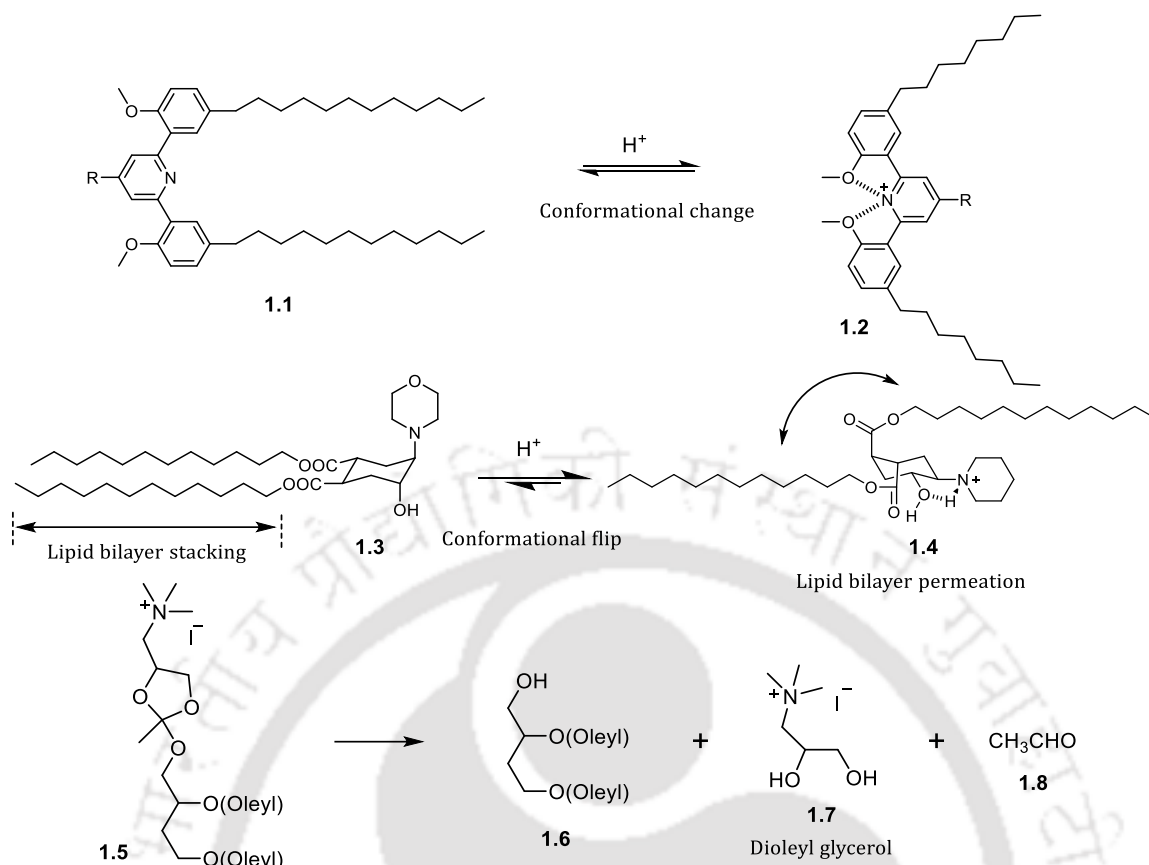


acidic, and extremely acidic intracellular compartments with a pH value of 5.5 are known as the pH-responsive release mechanism.<sup>79</sup> The microenvironment of a tumor is acidic because of the formation of lactic acid due to the cellular metabolism switching to glycolysis during the aggressive tumor cell division. This specific phenomenon is called the Warburg effect.<sup>80</sup>

**Table 1.1.** The pH of compartment/tissue of the human body.<sup>79</sup>

| Cellular Compartment/Tissue | pH        |
|-----------------------------|-----------|
| Early endosome              | 6.0–6.5   |
| Late endosome               | 5.0–6.0   |
| Lysosome                    | 4.5–5.0   |
| Golgi                       | 6.4       |
| Stomach                     | 1.0–3.0   |
| Duodenum                    | 4.8–8.2   |
| Colon                       | 7.0–7.5   |
| Blood                       | 7.35–7.45 |
| Tumor                       | 6.5–7.2   |

Lipid molecules with pH sensitivity in this acidic environment can be engineered by introducing a few functional groups such as hydrazone, imine, orthoester, amine, acetal, and vinyl ether, which consequently changes the chemistry either via enhanced solubility after protonation or bond cleavage of the material induces the bilayer destabilization and release the payload.<sup>81</sup> Viricel et al. reported that pH-triggered conformational change in lipid structure using di(methoxyphenyl)-pyridine-based lipids as the membrane switchable drug delivery.<sup>82</sup>



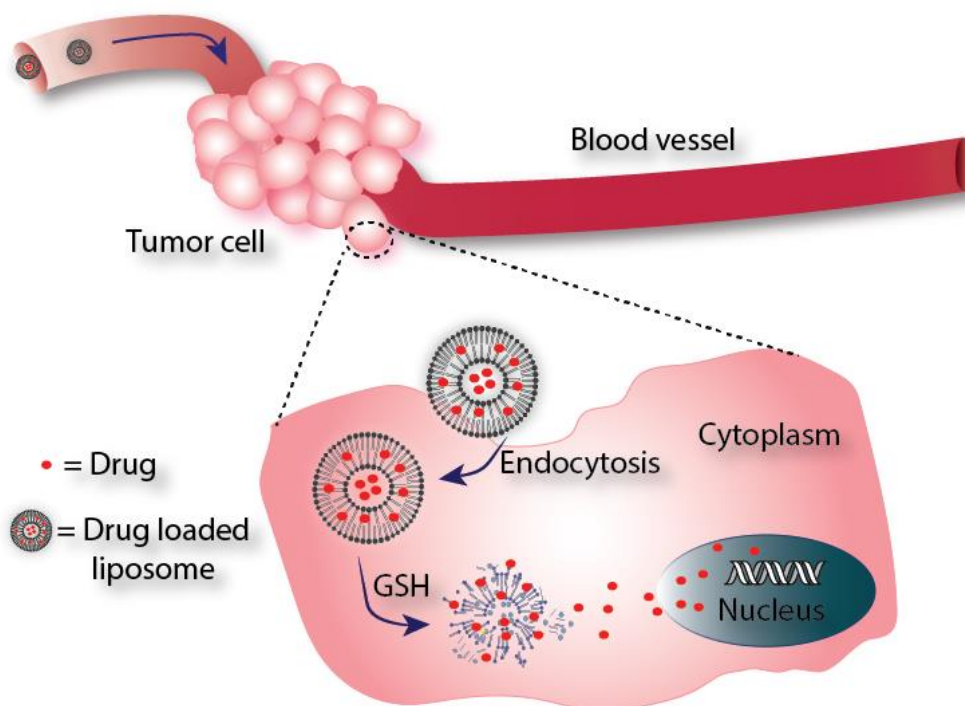
**Figure 1.6.** Reported pH-responsive lipid molecules.

Samoshina and co-workers developed liposomes by incorporating trans-2-morpholinocyclohexanol-based lipids into vesicle membranes that undergo a pH-sensitive conformational flip in acidic media.<sup>83</sup> Protonation of the morpholine ring induced conformational switch in the headgroups region produces a transient packing defect in the lipid tails, which triggers the release of payload.

#### 1.6.1.2. Redox-responsive delivery Strategy

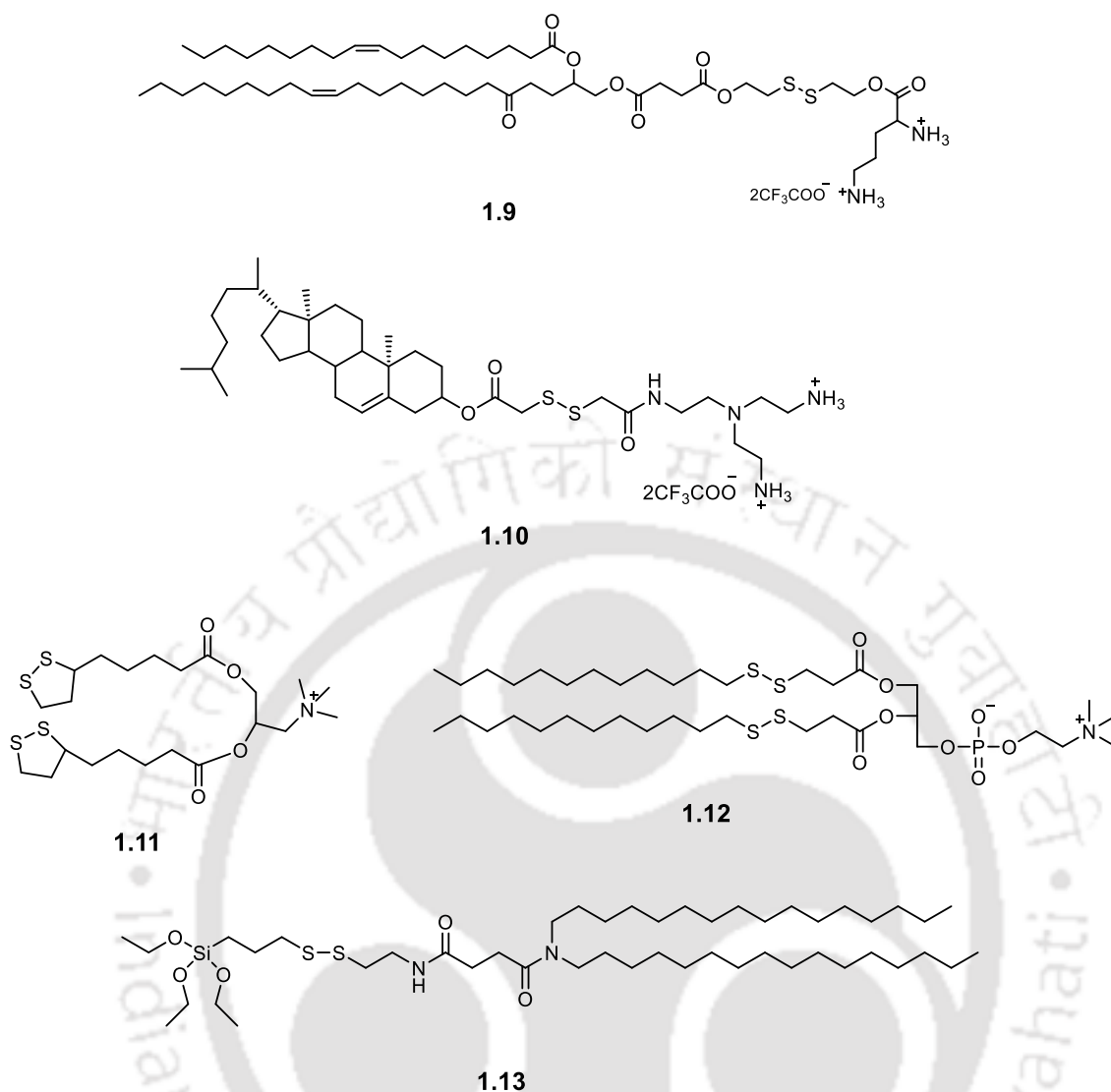
Recent research on redox-sensitive nanoparticles is a fairly exciting drug delivery strategy that relies on extracellular or intracellular redox molecules level for activation leading to endosomal escape or drug release. The reduced intracellular space and oxidizing extracellular environment of tumors function as a unique internal indicator that allows redox-responsive nanocarriers to degrade in tumor cells and release loaded cargoes.<sup>84</sup> Mainly, overexpression of GSH is responsible for the reductive nature of the cancer cell microenvironment. Notably, the concentration variation of GSH in the blood, as well as the environment of the tumor, is nearly 500-

fold.<sup>85</sup> Redox-responsive disulfide bonds containing liposomes have been extensively studied for the delivery system as disulfide bonds can be easily broken down by reducing GSH into sulfhydryl groups, which causes the degradation of carriers and facilitates the release of cargoes.<sup>86</sup>



**Figure 1.7.** Schematic representation of GSH-responsive anticancer drug delivery.

Zhang and co-workers report phospholipid conjugated with cleavable poly(ethylene glycol) (PEG) through a disulfide bond linker and its redox-sensitive showed a quick response of PEG detachment in a reductive microenvironment.<sup>86</sup> A very similar redox-responsive activity has been shown with diselenide bonds (Se–Se).<sup>87</sup> Jing Li and co-workers reported that linking the lipidic part of deoxycholic acid (DOCA) to the hydrophilic hyaluronic acid (HA) backbone via disulfide bonds can be used to synthesize the redox-responsive carrier HA-ss-DOCA for paclitaxel (PTX) targeted intracellular delivery.<sup>88</sup> Recently, it was reported that chitosan-based glycolipid-like nanocarrier (CSO-ss-SA) through the link between the hydrophobic structure and the hydrophilic backbone with disulfide bonds could be used in the co-delivery of siRNA and drugs.<sup>89</sup>

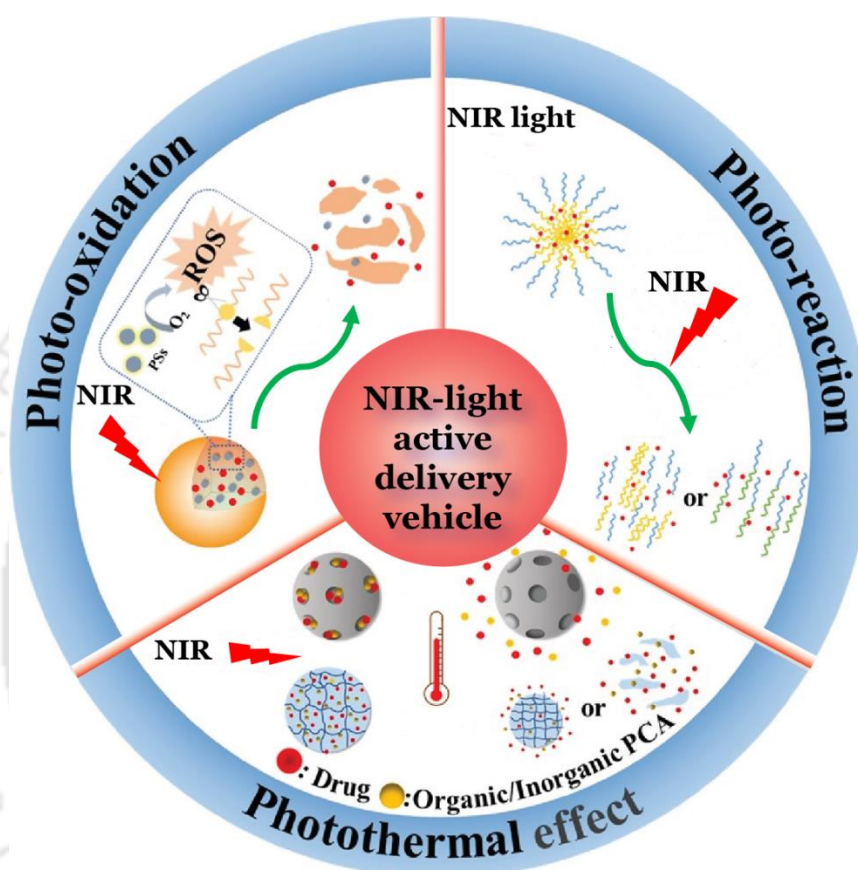


**Figure 1.8.** Few examples of GSH-responsive lipid molecules.

### 1.6.2. NIR light-responsive liposome for drug delivery

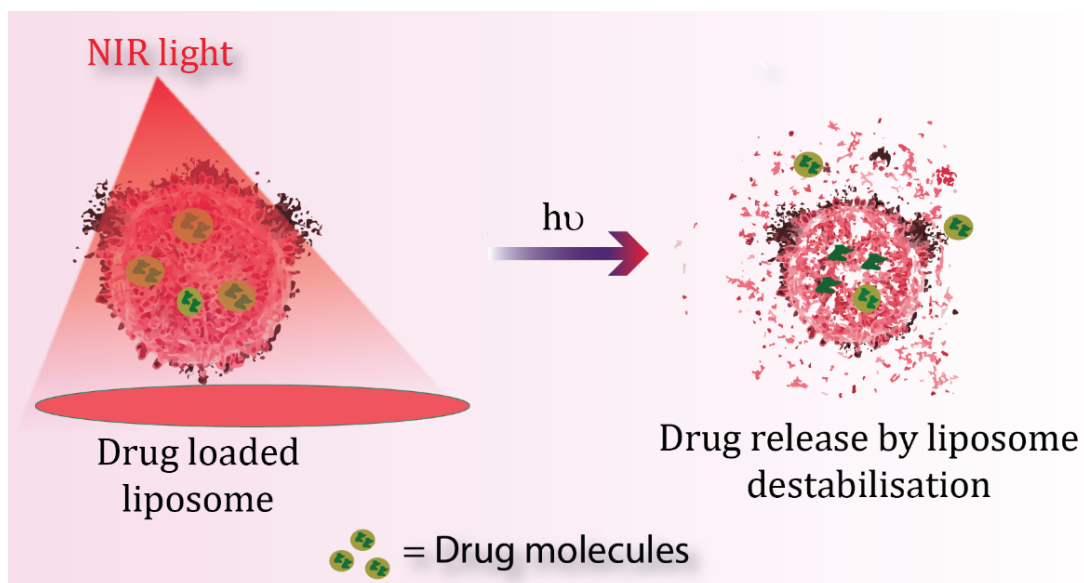
Numerous NIR light-responsive nanoplateforms have emerged in the past decades as a more attractive way to deal with external controllable therapeutics delivery. Light is particularly promising than other external stimuli (such as ultrasound, magnetic, and temperature) as it can be used over long distances with high temporal and spatial resolution.<sup>90</sup> NIR light-responsive nanocarriers for controlled release are principally based on the development of molecular-level photon absorptivity leading to structural change. For example, NIR-mediated chemical bond breaking, changes in molecular structure, and energy transfer to the adjacent molecule provide reference and guidance in designing NIR light-responsive nanocarriers for controlled release. However, three main triggering mechanisms of NIR light-responsive nanocarrier are

utilized for the NIR light-controlled release system: (1) bond cleavage or molecular configuration change of carrier molecule by the photoreaction of chromophoric functional group (such as 2-diazo-1,2-naphthoquinone (DNQ), coumarin, o-nitrobenzyl (ONB) and their derivatives)<sup>91</sup>; (2) Photothermal conversion by the organic or inorganic functional group under NIR light irradiation, which can

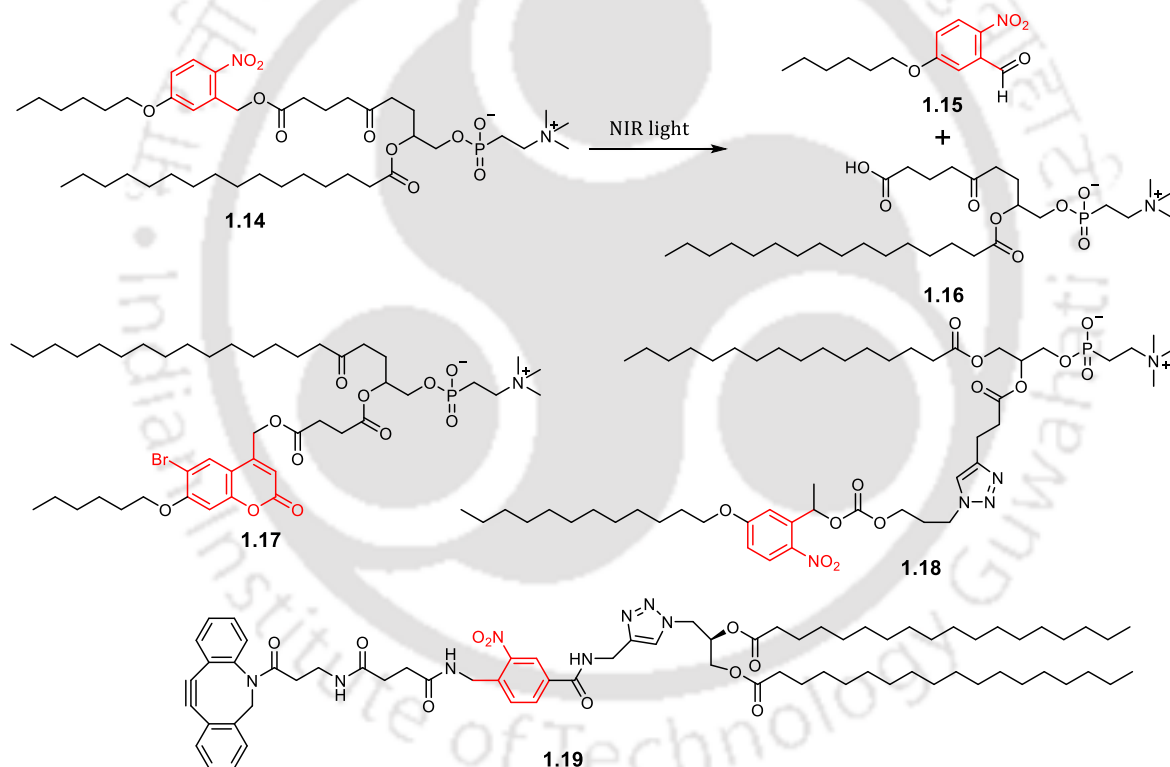


**Figure 1.9.** Schematic illustration of the drug release mechanism from NIR light-responsive nanocarrier induced by NIR light.

accelerate the diffusion of a loaded drug molecule and release to the specific sites<sup>92</sup>; (3) Photo-oxidation, NIR-light generated reactive oxygen species (ROS) by photosensitizers, and the ROS reacted with ROS-labile functional group of the delivery vehicle and disintegrated the nanocarrier.<sup>93</sup> The NIR light responsiveness via two-photon absorption (TPA) of nanocarriers is a fruitful strategy in terms of designing a lipid incorporating photoresponsive moiety.



**Figure 1.10.** Schematic representation of NIR light-mediated drug release.



**Figure 1.11.** Few light-responsive amphiphiles.

Yawei Sun and co-workers report that photoresponsive lipid with phosphatidylcholine head group can precisely control the release of loaded cargo by adjusting the light parameters.<sup>94</sup> Light mediated release of active drug molecule from the lipid-drug conjugate demonstrated by Shahrina Alam et al.<sup>95</sup> This promising strategy for convenient bioconjugation and NIR light-triggered release of bioactive



component and enhance the concentration of molecular cargo at the cell membranes of disease sites.

### **1.7. Development of targeted and fluorescence liposome for theranostic application**

Postfunctionalization of liposomes with a specific ligand strategy to target the cancerous cell is potentially promising, which minimizes the deposition of the drug molecule in the non-targeted tissues or with lesser off-target effects.<sup>96-97</sup> This approach involves tagging a targeting ligand to the surface of liposomes and selectively binding the targeted receptors on the diseased cells. Such liposomes are often called ligand-targeted liposomes or targeted liposomes, or immunoliposome.<sup>98-101</sup> Several receptors are over-expressed in cancer cells as compared to normal cells.<sup>102</sup> Targeting these over-expressed receptors is a dynamic approach for the active delivery pathway leading to higher uptake and accumulation of the anticancer drug in the cancer cell's microenvironment. Carbonic anhydrases (CA) are surface antigens (zinc metalloenzymes) found in red blood cells, which catalyze the bidirectional conversion of carbon dioxide ( $\text{CO}_2$ ) and water ( $\text{H}_2\text{O}$ ) into bicarbonate ( $\text{HCO}_3^-$ ) and protons ( $\text{H}^+$ ).<sup>103</sup> Two carbonic anhydrases enzyme CA-IX and CA-XII are found in higher amounts in many solid tumors due to hypoxia (lack of oxygen). CA-IX has shown higher activity than CA-XII in the cancer cell progression.<sup>104</sup> However, few ligands (such as acetazolamide) can effectively bind with the active pocket of the CA-IX enzyme and can restrict its function.<sup>105</sup> So, modification of liposome carrier with a ligand which selectively binds with CA-IX enzyme and enhances the local concentration of the drug molecule also simultaneously inhibits the functional enzyme. In the recent scenario, nano theranostics is an emerging frontier to diagnose and treat diseases at the cellular and molecular levels. Theranostics is a hybrid of therapeutics and diagnostics. Hence, the development of a targeted therapeutics carrier and imaging functionality has a better applicable potential for image-guided delivery and monitoring of therapeutic effects. The approach can provide the cancer treatment at the right time and in the right dosage with more efficiency and with minimized expenditures.

**Table 1.2.** Surface engineered liposomes functionalized with targeting ligands for solid tumor therapy

| Targeting ligand                      | Anticancer agent              | Targeting site |
|---------------------------------------|-------------------------------|----------------|
| Fab fragments of mAb C225 (cetuximab) | Doxorubicin                   | EGFR           |
| Anti EGFR antibody                    | Small interfering RNA (siRNA) | EGFR           |
| Anti HER2 antibody                    | Doxorubicin                   | HER2           |
| CA-IX Antibody                        | Docetaxel/ Doxorubicin        | CA-IX          |
| Anti-MT1-MMP Fab                      | Doxorubicin                   | MT1-MMP        |
| Trastuzumab (Anti HER2)               | Paclitaxel and Rapamycin      | HER2           |
| Anti VEGFR2                           | Doxorubicin                   | VEGFR2         |

### 1.8. Summary

The therapeutic benefit of liposomes in assisting drug delivery significantly impacts many biomedical areas. Several liposome-based nanomedicines have been successfully developed for clinical applications of cancer, gene delivery, infection, imaging, vaccine, and others. Recent studies on stimuli-responsive liposomes showed a promising approach for cargo release on the tumor tissues. However, limitations in pharmaceutical development of the formulation in manufacturing scale, stability, and targetability are still issues. The real challenges that arise in integrating multiple components to a single nanocarrier involve multiple chemical synthesis steps and formulation processes that increase the number of physicochemical parameters of the additional variables in the formulation system and make it more complicated for the pharmacokinetics, pharmacodynamics, and toxicology of a formulation. Henceforth, synthesis of the lipid molecule with practical measures of diverse functional groups is always demanding in the arena of targeted drug delivery to advance the disease's treatment.

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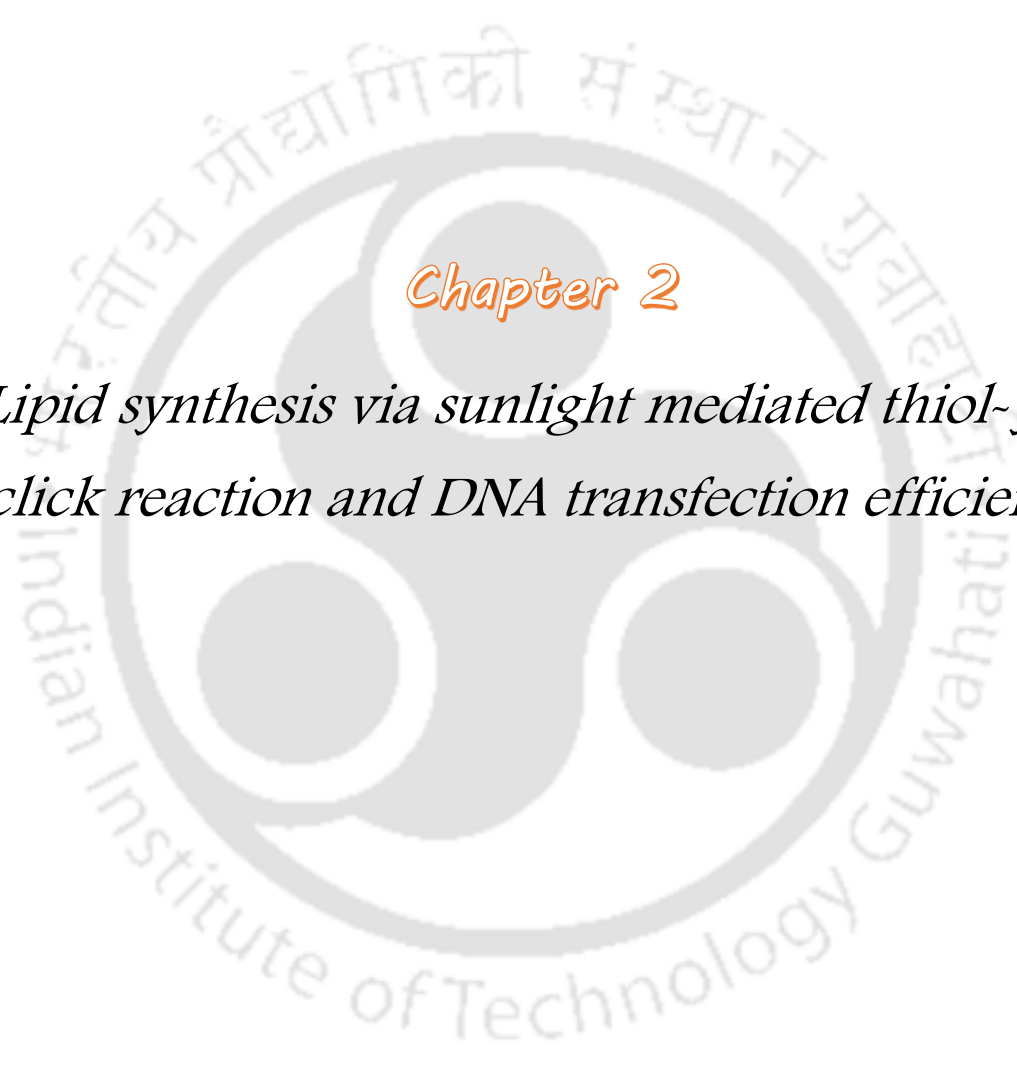
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The background of the slide features a large, faint watermark of the Indian Institute of Technology Guwahati logo. The logo is circular, containing a stylized 'IIT' monogram in the center. The text 'Indian Institute of Technology Guwahati' is written in English around the bottom half of the circle, and its Assamese equivalent 'গুৱাহাটীৰ ভাৰতীয় প্রযুক্তিগতীকী সংস্থান' is written along the top half.

## Chapter 2

*Lipid synthesis via sunlight mediated thiol-yne  
click reaction and DNA transfection efficiency*



## 2.1. Background and objective of present work

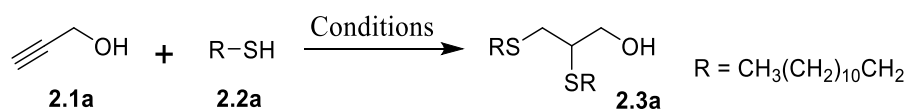
The concise literature on synthetic lipids in chapter 1 evidently demonstrates its potential for therapeutics delivery, primarily in cancer cells and other diseases. The design of lipid molecules and synthesis in a milder approach plays a crucial role and is highly rewarding. Over the past two decades, numerous viral or nonviral gene delivery systems have been developed to fight against various life-threatening diseases, including cancer, cardiovascular alignment, and others. Clinical studies have shown that the modification of cells at the genetic level significantly improves their therapeutic potential.<sup>1-2</sup> However, a carrier is required to encapsulate and transport the negatively charged genetic materials across the hydrophobic lipid bilayer for its successful delivery to the cells. Viral vector-based delivery systems have been widely used to successfully deliver genetic materials both under *in vitro* and *in vivo* conditions. But, the random integration of the viral gene delivery system into the host genome interposes the target gene expression, which diminishes their therapeutic potential. In this regard, nonviral gene delivery systems have been developed as an alternative approach.<sup>1, 3-7a</sup> Among the nonviral gene delivery systems, natural/synthetic lipids are one of the most promising vehicles to optimize the delivery of exogenous genetic materials.<sup>8-9</sup> The lipids provide a safeguard to genetic materials and obtain improved bioavailability with reduced immunogenicity and cytotoxicity. In particular, the cationic lipid-based vehicles show tremendous applications because of their complexation ability with negatively charged genetic materials to form efficient lipoplexes and membrane fusion capability.<sup>10-11</sup> Since the discovery of *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) (a cationic lipid with ammonium headgroup and with ether-linked hydrophobic tails), several lipids have been developed to augment the delivery of genetic materials.<sup>11-15</sup> Few cationic lipid-based gene delivery vehicles are also commercially available (such as Lipofectamine 2000 and TransFast).<sup>16</sup> However, the gene delivery efficacy of most of the reported cationic lipids is generally not adequate for clinical and other *in vivo* applications and also has shortcomings, including harsh reaction conditions with multistep synthetic routes, structural complexity, and low stability, low transfection efficiency, and biocompatibility. Recent studies also demonstrated that the higher numbers of cationic ammonium moieties in the headgroup region also enhance their cytotoxic effect.<sup>16-21</sup>

So encouraged by all these therapeutic prospects of cationic lipids for gene delivery, the focus was given to the design and synthesis of cationic lipids via a milder approach. In this chapter, we demonstrate a sunlight-mediated thiol-ene/yne click reaction in the presence of a very few amount of tert-butyl hydroperoxide (TBHP) in the aqueous medium. The optimized reaction conditions have been proven to be efficient and powerful for the synthesis of small molecules of chemical and pharmaceutical interest. This synthetic methodology was also applied to prepare a series of modular lipid molecules with neutral, zwitterionic, and cationic headgroups. After synthesis, a thorough investigation of nanoaggregates (NAs) formation, DNA complexation affinity, serum stability, and several other biophysical experiments were carried out. Besides that, a number of *in vitro* assays were also performed to prove its transfection efficiency.

## 2.2. Results and discussions

### 2.2.1. Optimization of the Reaction Conditions for the Thiol-Yne Click Reactions under Aqueous Environment

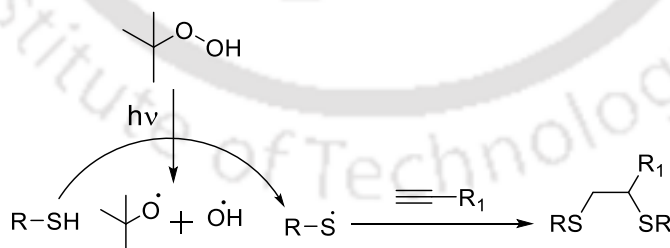
We started with mild reaction conditions to perform the thiol-ene/yne click reaction in an aqueous medium. The thiyl radicals are known to undergo click reactions with alkenes or alkynes in the absence or presence of radical initiators under an organic solvent.<sup>4</sup> However, the formation of thiyl radicals in the aqueous medium is challenging. Hence, our first target was to generate thiyl radicals in the aqueous medium. We started our investigation with the model reaction of propargyl alcohol and dodecanthiol under sunlight in various reaction conditions, and the results are summarized in Table 2.1. The tert-butyl hydroperoxide (TBHP) is a water-soluble, inexpensive radical initiator and known to form t-BuO• or t-BuOO• radicals in the aqueous medium.<sup>22</sup> The oxidation of thiol by t-BuO• or t-BuOO• radicals could generate the thiyl radicals that can react with alkene/alkyne to produce the thioethers. To our delight, a very small amount of TBHP (0.05 mol %) as the radical initiator and H<sub>2</sub>O as the solvent (Table 2.1, entry 17) led to the formation of the hydrothiolated product with the highest yields. Interestingly, redox additives, including Fe<sup>2+</sup>/Fe<sup>3+</sup> and Cu<sup>+</sup>/Cu<sup>2+</sup>, adversely affected the model reaction with lower yields. The use of H<sub>2</sub>O<sub>2</sub> and di-tert-butyl peroxide (DTBP) failed to produce the targeted product. Solvent screenings revealed that H<sub>2</sub>O or H<sub>2</sub>O/DMF (1:0.01) was

**Table 2.1.** Optimization of the reaction conditions for the synthesis of thioethers via thiol-yne click reaction in the aqueous medium.

| Entry     | Solvent <sup>a</sup> | Initiator<br>(mol %)          | Additive<br>(mol %)                     | External<br>stimulus | Time<br>(min) | Yields<br>(%) <sup>b</sup> |
|-----------|----------------------|-------------------------------|-----------------------------------------|----------------------|---------------|----------------------------|
| 1         | H <sub>2</sub> O     | TBHP (1.0)                    | FeCl <sub>3</sub> (0.5)                 | Sunlight             | 30            | 77                         |
| 2         | H <sub>2</sub> O     | TBHP (1.0)                    | FeSO <sub>4</sub> (0.5)                 | Sunlight             | 30            | 71                         |
| 3         | H <sub>2</sub> O     | TBHP (1.0)                    | Fe(NO <sub>3</sub> ) <sub>3</sub> (0.5) | Sunlight             | 30            | 72                         |
| 4         | H <sub>2</sub> O     | TBHP (1.0)                    | CuCl (0.5)                              | Sunlight             | 30            | 78                         |
| 5         | H <sub>2</sub> O     | -                             | FeCl <sub>3</sub> (0.5)                 | Sunlight             | 30            | ND                         |
| 6         | H <sub>2</sub> O     | -                             | FeSO <sub>4</sub> (0.5)                 | Sunlight             | 30            | ND                         |
| 7         | H <sub>2</sub> O     | -                             | Fe(NO <sub>3</sub> ) <sub>3</sub> (0.5) | Sunlight             | 30            | ND                         |
| 8         | H <sub>2</sub> O     | -                             | CuCl (0.5)                              | Sunlight             | 30            | ND                         |
| 9         | H <sub>2</sub> O     | TBHP (1.0)                    | -                                       | Sunlight             | 30            | 85                         |
| 10        | H <sub>2</sub> O     | H <sub>2</sub> O <sub>2</sub> | -                                       | Sunlight             | 30            | 10                         |
| 11        | H <sub>2</sub> O     | DTBP                          | -                                       | Sunlight             | 30            | 80                         |
| 12        | DMF                  | TBHP (1.0)                    | -                                       | Sunlight             | 30            | 91                         |
| 13        | THF                  | TBHP (1.0)                    | -                                       | Sunlight             | 30            | 92                         |
| 14        | Dioxane              | TBHP (1.0)                    | -                                       | Sunlight             | 30            | 89                         |
| 15        | H <sub>2</sub> O     | TBHP (0.5)                    | -                                       | Sunlight             | 30            | 83                         |
| 16        | H <sub>2</sub> O     | TBHP (0.1)                    | -                                       | Sunlight             | 30            | 83                         |
| 17        | H <sub>2</sub> O     | TBHP (0.05)                   | -                                       | Sunlight             | 30            | 91                         |
| 18        | H <sub>2</sub> O     | TBHP (0.05)                   | -                                       | UV light             | 30            | 90                         |
| 19        | H <sub>2</sub> O     | TBHP (0.05)                   | -                                       | dark                 | 30            | < 10                       |
| condition |                      |                               |                                         |                      |               |                            |
| 20        | H <sub>2</sub> O     | TBHP (0.05)                   | TEMPO (0.5)                             | Sunlight             | 30            | ND                         |

<sup>a</sup>The reaction was performed using 0.46 mmol of **2.1a** (1 equiv) and 1.0 mmol of **2.2a** (2.2 equiv) under sunlight. <sup>b</sup>Yields of purified compounds. ND = not detected. DMF = *N,N*-dimethylformamide. THF = tetrahydrofuran.

producing very similar results to those in the presence of organic solvents (Table 2.1, entries 12–14 and 17). Therefore, to develop mild reaction conditions, H<sub>2</sub>O or H<sub>2</sub>O/DMF solvent mixture was used. The reaction was also successfully performed in the presence of UV light ( $\lambda_{\text{ex}} = 365 \text{ nm}$ , 1W), suggesting that the reaction can also be performed in the absence of sunlight. Additionally, the reaction under dark conditions produces the targeted compound with a very low yield, which indicates that light is necessary for the reaction. Moreover, a control reaction in the presence of (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) drastically reduced the reaction yield (Table 2.1, entry 20), which confirms that the reactions proceed through the radical pathway under the aqueous environment. The thiols and alkenes/alkynes are generally unreactive toward each other. However, various initiators can initiate their reactions. We hypothesized that the coupling of thiols with alkyne proceeds via a radical reaction pathway, and the TBHP acts as a thiyl radical initiator under an aqueous medium. The exposure to sunlight initiates the cleavage of TBHP into tert-butoxyl and hydroxyl radicals.<sup>22</sup> Subsequently, the tert-butoxyl radical converts the thiols into thiyl radicals, and these thiyl radicals undergo a coupling reaction with alkynes to produce the corresponding hydrothiolated product. The formation of tert-butoxyl and hydroxyl radicals under the optimized reaction conditions was confirmed by the high-resolution mass spectrometry (HRMS) analysis of the reaction mixture (Figure 2.42). Based on these experimental outcomes and control experiments, a proposed mechanism for this TBHP-initiated thiol-ene/yne click reaction is demonstrated in (Figure 2.1)



**Figure 2.1.** The proposed mode of reaction under optimized reaction conditions.

### 2.2.2. Synthesis of Small Molecules and Cationic Lipids

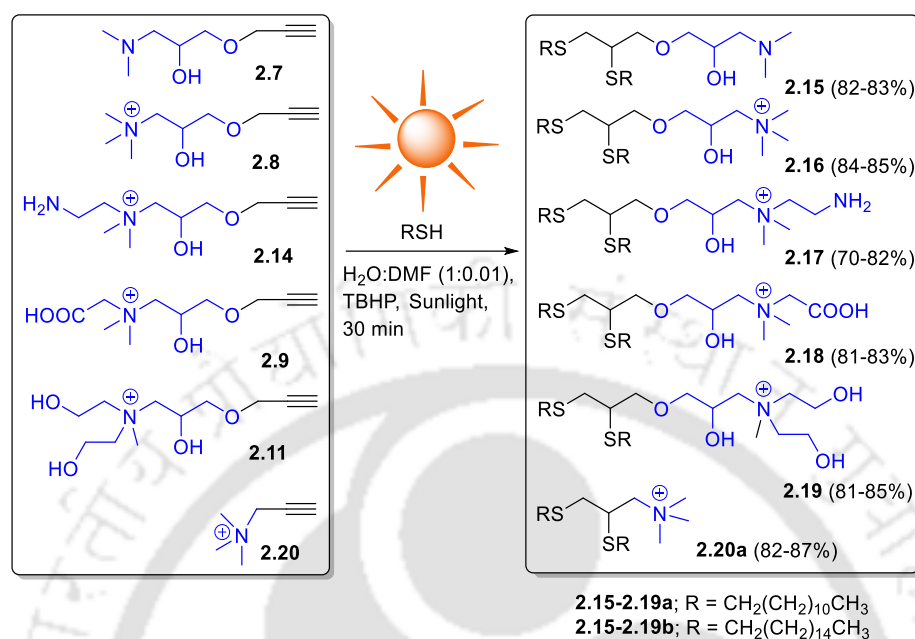
With the optimized reaction conditions in hand, we explored differently substituted alkenes and alkynes with various thiols for the application of this thiol-ene/yne click reaction under an aqueous medium (Table 2.3). The reaction of



aliphatic alkynes with thiols showed bis-hydrothiolation reactions with an excellent yield of 89–92% (compounds **2.3a–2.3c**). However, the anti-Markovnikov selective thiol-yne click reaction showed a monohydrothiolated product from aromatic alkynes (compounds **2.3d–2.3f**). The aliphatic and aromatic alkenes also showed excellent hydrothiolation reactions under the optimized reaction conditions (compounds **2.5a–2.5g**).

We also synthesized a series of lipids using the thiol-ene/yne click reaction under the optimized reaction conditions. We synthesized three different types of lipids with cationic, zwitterionic, and neutral headgroups.<sup>23-25</sup> The headgroups of lipid molecules were synthesized from a common starting molecule 2-((prop-2-yn-1-yloxy)methyl)oxirane (Scheme 2.1). The reaction of 2-((prop-2-yn-1-yloxy)methyl)oxirane (**2.6**) with the mono-/di-alkyl amine, followed by methylation of amine, resulted in the headgroup of the lipid molecules. The reaction of dimethylamine with 2-((prop-2-yn-1-yloxy)methyl)oxirane (**2.6**) resulted in alkynyl derivative 1-(dimethylamino)-3-(prop-2-yn-1-yloxy)propan-2-ol (**2.7**), which upon reaction with iodomethane and 2-bromoacetic acid yielded alkynyl derivatives **2.8** and **2.9**, respectively. The reaction of compound **2.6** with 2,2'-azanediyl diethanol, followed by methylation, resulted in alkynyl derivative **2.11**. The reaction of compound **2.6** with tert-butyl (2-aminoethyl)carbamate, followed by methylation and removal of the Boc group, produced the alkynyl derivative **2.14** (Scheme S1). After that, the reactions between these alkynyl headgroups with long-chain alkyl thiols were performed by using our optimized reaction conditions. The reactions of alkyl thiols of different chain lengths (C12 and C16) with alkynyl derivatives produced lipids **2.15–2.19** (Figure 2.2). We also synthesized the control compound **2.20a** (without the  $\beta$ -hydroxy group in the linker region) under similar reaction conditions (Figure 2.2). Interestingly, the thiol-yne reaction produced the thioether-based lipids in the absence of protecting groups, suggesting that the presence of free acid, amine, and hydroxyl groups did not appear to obstruct the formation of lipids. The higher chemoselectivity of the thiol-yne reaction could be one of the reasons for the efficient synthesis of these lipids.<sup>25</sup> It is important to mention that for the synthesis of compounds **2.15–2.20**, the H<sub>2</sub>O/DMF solvent mixture (1:0.01) was used to increase the solubility of dodecanethiol or hexadecanethiol. However, the reaction may also proceed at the interface of the aqueous medium. Overall, we successfully

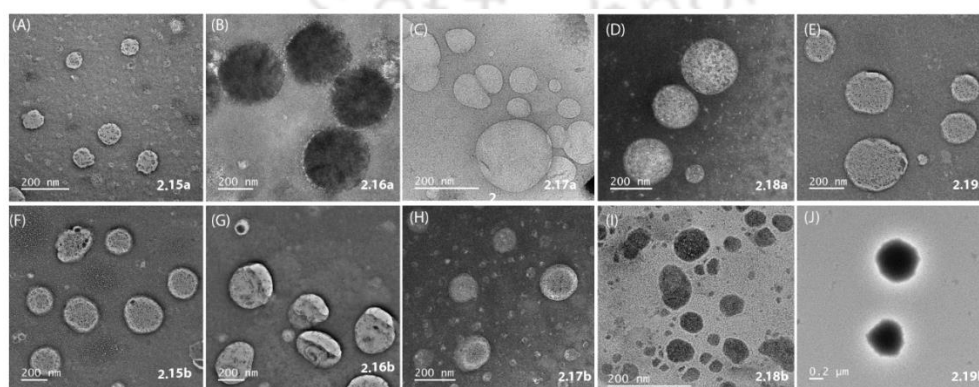
synthesized a series of lipids using an inexpensive and environment-friendly approach.



**Figure 2.2** Rapid synthesis of dithioether-based cationic lipids through sunlight-mediated thiol-yne clicks reactions in the aqueous medium.

### 2.2.3. Morphology of the lipid vesicles

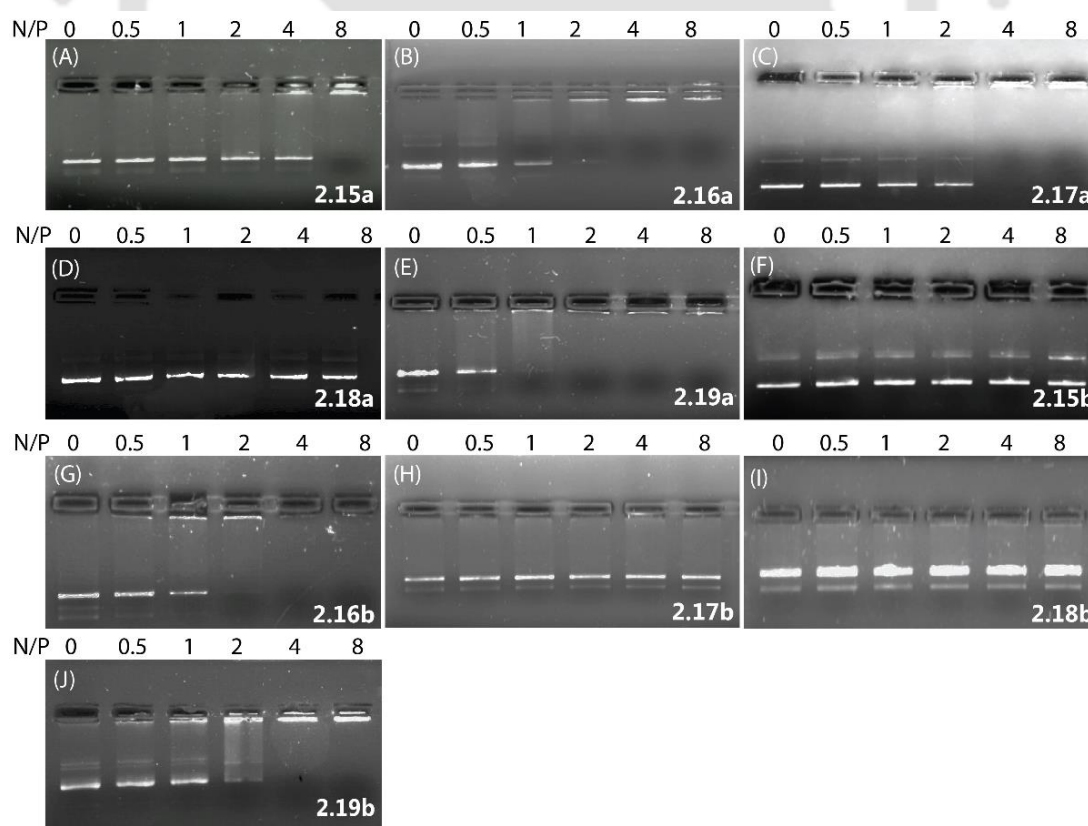
The aqueous phase morphology of the synthesized lipids was investigated by Transmission Electron Microscopy (TEM) analyses. We observed that hydration of the thin films of the lipids forms stable aggregates in an aqueous solution.<sup>26-28</sup> The TEM images revealed the formation of small and medium-sized liposomes (200–450 nm) by these lipids (Figure 2.3).



**Figure 2.3.** TEM images of NAs of lipids 2.15-2.19a (A-E) and 2.15-2.19b (F-J).

### 2.2.4. Interaction of the Lipids with the Plasmid DNA

The liposomes of these synthetic lipids were prepared in combination with co-lipid PE (1:1 mole ratio). The inverted hexagonal structure of PE lipid is known to facilitate the endosomal membrane fusion and facile release of DNA, leading to higher transfection efficiencies of the lipoplexes.<sup>29-30</sup> The DNA-binding capability is the prerequisite for gene delivery vectors, and the optimum balance between the DNA binding and release is imperative to enhance transfection efficiency. The DNA binding ability of these liposomes was investigated by electrophoretic gel retardation assay. The green fluorescence protein (GFP)-tagged plasmid DNA (bp = 5kb) of the MMP7 enzyme (pMMP7-GFP) was used for all gene delivery-related studies. Efficient gene delivery vehicles should bind plasmid DNA at low N/P ratios. The outcome of the gel retardation studies showed that both hydrophobic chain length and overall charge of the headgroup have a variable effect on their DNA-binding ability (Figure 2.4).



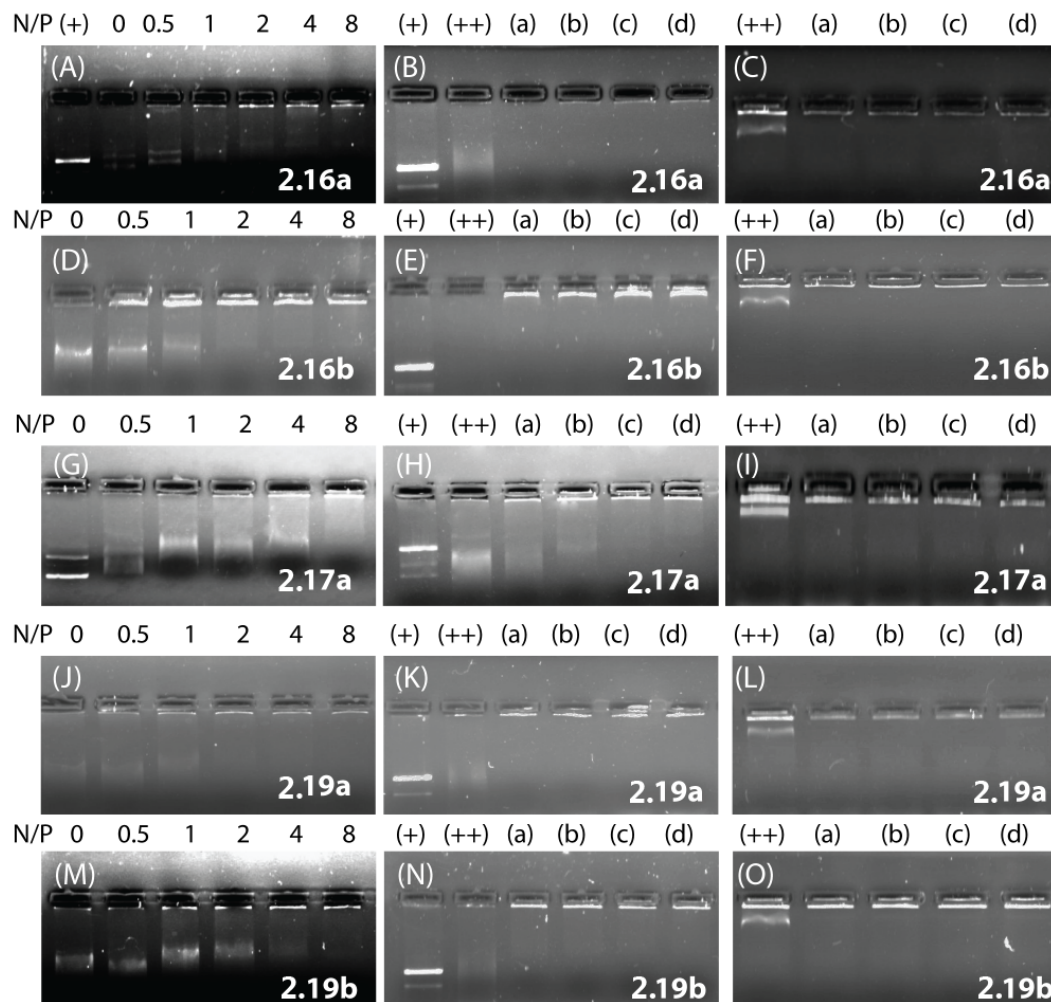
**Figure 2.4.** Electrophoretic gel retardation assay of pMMP7-GFP in the absence and presence of co-liposomes at different N/P ratios (0 to 8). The molar ratio of synthetic

lipid and PE was 1:1 (mole ratio). All the gel electrophoresis measurements were performed in triplicates.

The mobility of the plasmid DNA was strongly inhibited in the presence of the lipids with monocationic headgroups (**2.16a** and **2.19a**) at the N/P ratios of 1–2. The lipids with zwitterionic headgroup (**2.18a** and **2.18b**) failed to retard the plasmid DNA up to the N/P ratio of 8, indicating their weaker DNA binding ability. The cationic and polyamine-containing lipids are known to have stronger DNA-binding ability.<sup>8, 31</sup> Surprisingly, the lipids **2.17a** and **2.17b** with ammonium and trialkylammonium headgroups showed lower plasmid DNA-binding ability (N/P ratio  $\geq 2$ ), suggesting that the presence of the  $\beta$ -hydroxy group in the linker region plays a vital role in their DNA-binding abilities. The hydroxy group may interact with the terminal ammonium group of lipid, leading to its inaccessibility for DNA binding.<sup>15</sup> We also synthesized lipid **2.20a** without the  $\beta$ -hydroxy group-containing linker region. The gel retardation studies showed a much weaker DNA-binding ability (even N/P > 4) of lipid **2.20a**, suggesting the importance of the  $\beta$ -hydroxy group in DNA binding (**Figure 2.43**). Overall, the cationic lipids showed stronger DNA-binding abilities with C12 in comparison to that of C16 chain length, suggesting that the length of the hydrophobic alkyl group is also a key parameter in stable lipoplex formation. Hence, the plasmid DNA-binding affinity of the synthesized lipids follows an order of **2.19a**  $\approx$  **2.16a**  $\approx$  **2.16b** > **2.19b**  $\approx$  **2.17a** > **2.15a** > **2.15b**  $\approx$  **2.17b**  $\approx$  **2.18a**  $\approx$  **2.18b**, suggesting that the DNA-binding affinity of the lipids can be tuned by choosing certain types of headgroup and hydrophobic tails. The lipoplexes should be stable enough for cellular applications to evade possible detachment under physiological conditions before they reach the targeting tissues. In this regard, the DNA-binding ability of the potent cationic lipids **2.16** and **2.19** was investigated in the presence of serum.<sup>16</sup> The gel retardation assay showed that these cationic lipids also effectively inhibited the mobility of plasmid DNA at N/P ratio of 2–4 in the presence of both 10 and 50% serum, suggesting that the presence of serum has a minimal effect on their DNA-binding abilities (**Figure 2.5**). The time-dependent studies showed no release or enzymolysis of DNA even after 2 h, suggesting the stability of the lipoplexes in the presence of serum (**Figure 2.5**). It is important to mention that the appearance of one or two bands with different brightnesses in the gel images could be due to the change



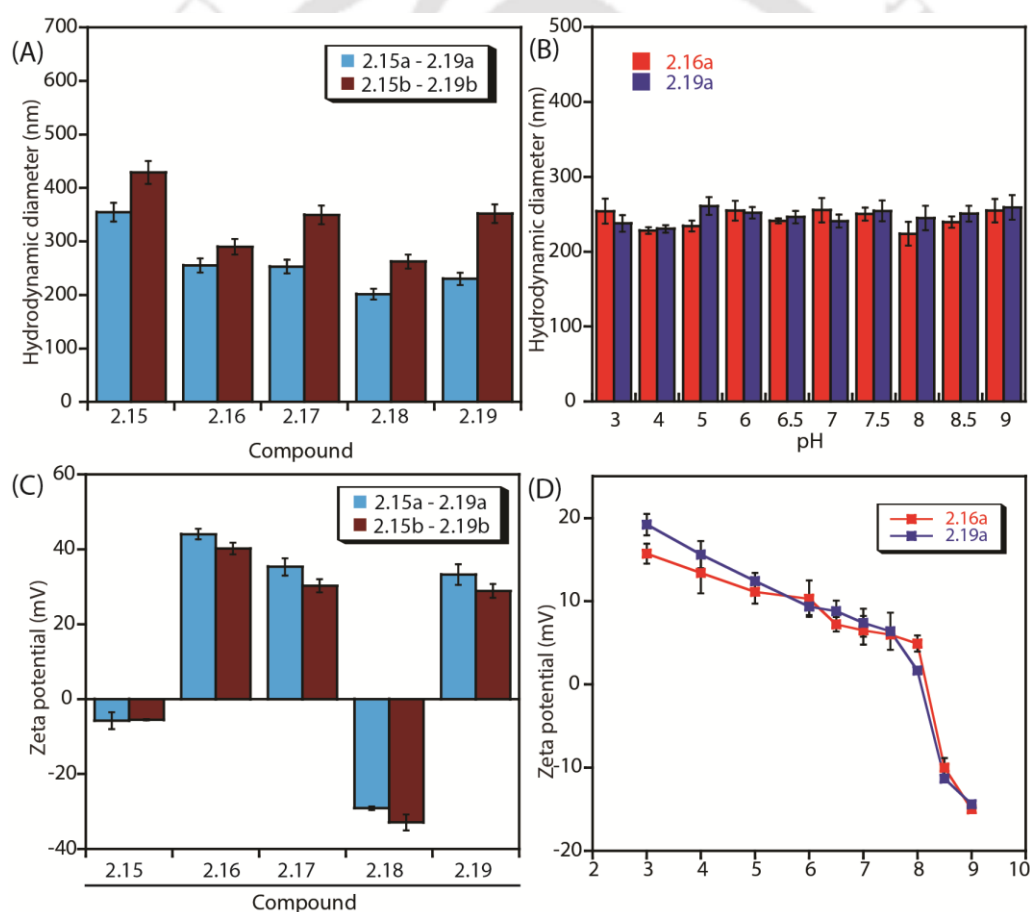
in the structure of the DNA after the formation of the lipoplexes. From the above experimental results, we can conclude that compounds **2.16** and **2.19** showed better lipoplex formation capability.



**Figure 2.5.** Electrophoretic gel retardation assay of pMMP7-GFP in the presence of coliposomes at different N/P ratios (A, D, G, J and M). The molar ratio of synthetic lipid and PE was 1:1. Time-dependent electrophoretic gel retardation assay of lipid **2.16a** (B), **2.16b** (E), **2.17a** (H), **2.19a** (K) and **2.19b** (N) at N/P ratio of 2 in the presence of 10% serum. Time-dependent electrophoretic gel retardation assay of lipid **2.16a** (C), **2.16b** (F), **2.17a** (I), **2.19a** (L) and **2.19b** (O) at N/P ratio of 2 in the presence of 50% serum. (+) = DNA without serum, (++) = DNA with serum, (a) = 0 minute, (b) = 30 minutes, (c) = 60 minutes, (d) = 120 minutes.

### 2.2.5. Zeta potential and hydrodynamic diameter of the lipid vesicles

We characterized the particle size and surface potential of the lipids in an aqueous environment. The dynamic light scattering (DLS) measurements also showed the formation of vesicles in an aqueous medium with a very similar hydrodynamic diameter ( $d_H$ ) and narrow size distribution (Figure 2.6A). The hydrodynamic diameters of the liposomes obtained by the DLS measurements of the lipids were found to be within 200–450 nm. The lipids with shorter chain lengths formed liposomes with smaller sizes. We also examined the effect of pH on the hydrodynamic diameter and surface charge of the lipids. The DLS measurements showed that the pH of the environment has little impact on the hydrodynamic diameter of the lipids **2.16a** and **2.19a** (Figure 2.6B).<sup>16</sup> However, the pH of the



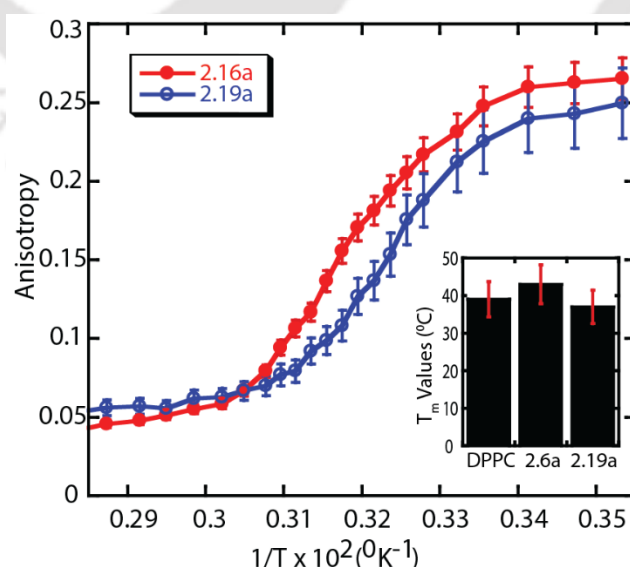
**Figure 2.6.** Mean particle size (A) and surface potential (C) of the lipid vesicles at pH 7.4. Mean particle size (B) and surface potential (D) of the lipids **2.16a** and **2.19a** at different pH solutions. All measurements were performed at room temperature.



environment plays a vital role in the surface charge of the lipids, which could be due to the presence of ammonium ions in the lipid headgroup (Figure 2.6C). The intramolecular ionic interaction could be one of the reasons for the deprotonation of secondary alcohol around pH 8.0. The isoelectric points (IEPs) of the lipids **2.16a** and **2.19a** were 8.19 and 8.09, respectively. The IEP values also demonstrate that these lipids are generally cationic under physiological conditions.

### 2.2.6. Phase transition temperature measurements

Fluidity and the transition from the gel phase to the liquid disordered phase are essential parameters of the lipids. Hence, the phase-transition temperature ( $T_m$ ) of the cationic lipids can be considered a fundamental physical property for investigating the thermal stability and gene delivery properties of the liposomes. We performed temperature-dependent steady-state anisotropy measurements using the membrane-embedded 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence probe to determine the  $T_m$  values of the lipids (Figure 2.7). The observed  $T_m$  values of the two potent lipids **2.16a** and **2.19a** were 43 and 37 °C, respectively. This elevation of  $T_m$  values could be due to the more ordered packing in the plane of the lipid bilayer. This moderate thermal stability also suggests that these lipids could have multiple biological applicability. We also assume that the liposomes would also be stable in the presence of fusogenic co-lipids such as 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (PE).



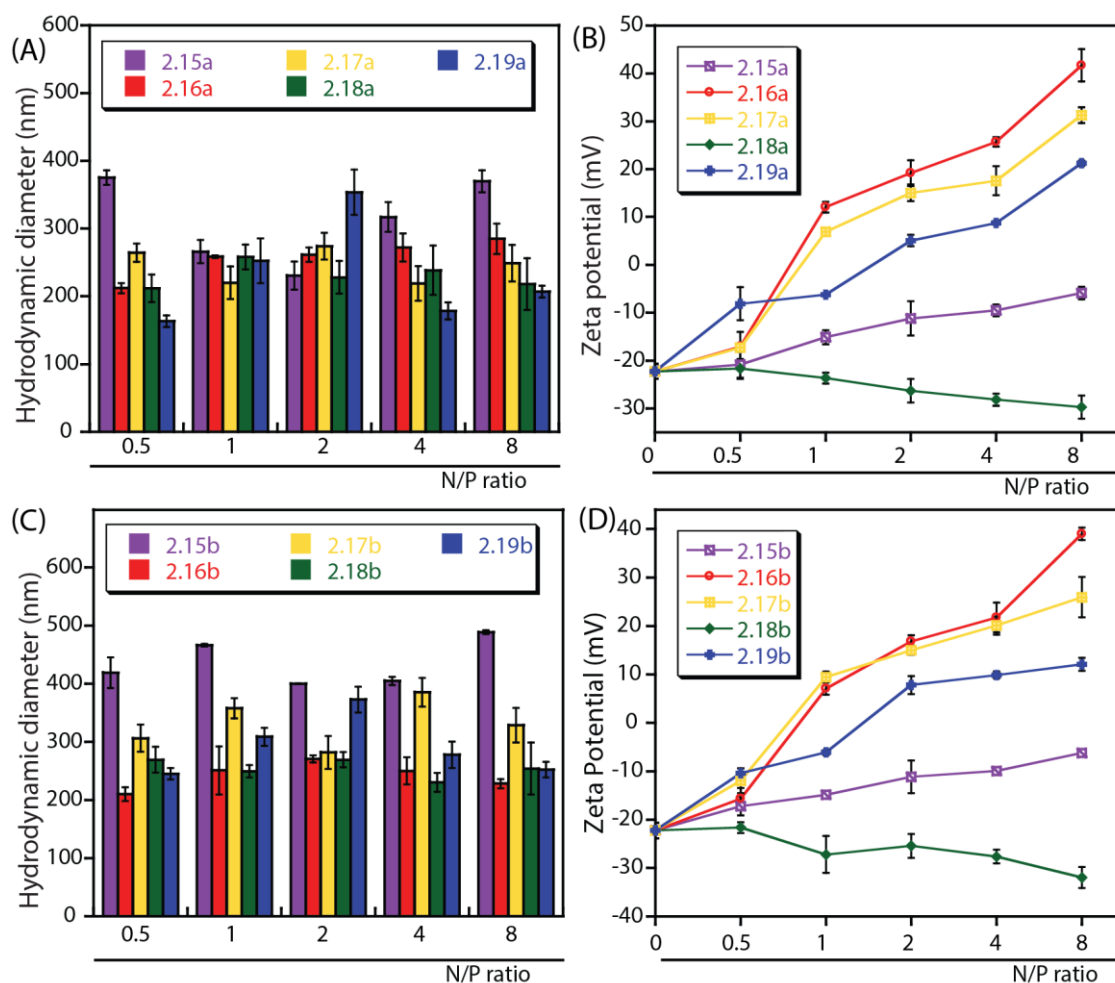
**Figure 2.7.** Temperature-dependent steady-state fluorescence anisotropy measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) under the vesicle

environment for **2.16a** and **2.19a** lipids within the range of 10–80 °C (D). The inset shows the  $T_m$  values of the lipids.

### 2.2.7. Relation between the Particle Size and Surface Charge of the Lipoplexes

The gene transfection efficiency greatly depends on the size and surface charge of the lipoplexes, which depends on the structural properties of the headgroup and hydrophobic tails. In this regard, the hydrodynamic diameter ( $d_H$ ) and surface charge of the lipoplexes were investigated. The outcome of the DLS measurements showed that the average  $d_H$  of the liposomes and lipoplexes was within 200–500 nm (Figure 2.8A, C). The variable sizes of the liposomes suggest that the hydrophilic properties of the headgroup played an essential role in liposome formation. The lipids with shorter chain lengths could produce lipoplexes with a small size. The liposomes of **2.16**, **2.17**, and **2.19** have a positive surface charge, facilitating their electrostatic interaction with the negatively charged DNA. The lipids **2.15** and **2.18** formed negatively charged liposomes under physiological conditions. Meanwhile, different surface charges were observed for the lipoplexes at various N/P ratios (Figure 2.8B, D). The lipoplexes of **2.16**, **2.17**, and **2.19** demonstrated a rapid increase in the surface charge when the N/P ratios varied from 0.5 to 8. The lipoplexes of **2.16** and **2.19** have a cationic surface potential, and average  $d_H$  values were less than 400 nm at an N/P ratio of 2, at which full retardation of DNA was observed in the gel electrophoresis assay. The hydrophobic chain length had no substantial effect on both size and surface charge of the lipoplexes. The lipoplex of **2.15** showed a slower increase in surface charge, but the lipoplex of **2.18** showed a decrease in surface charge within the same range of N/P ratios, suggesting that the surface charge could be tuned by the selection of a particular lipid structure. The outcome of the size and surface charge measurements suggests that the lipoplexes might possess a different cellular gene transfection efficiency. The TEM analysis was also performed to investigate the morphologies of the lipoplexes at an N/P ratio of 2 (Figures 2.44 and 2.45). The TEM images showed that both the lipids could adequately encapsulate the DNA. However, the size of the lipoplexes obtained from the TEM images of **2.16** and **2.19** (50–200 nm) is much smaller than that measured by DLS analysis (200–400 nm), which could be due to the shrinking of lipoplexes in the drying process during the TEM sample preparation, while the DLS measures the hydrodynamic sizes in the

aqueous solution. The lipoplexes formed from lipid **2.16a** showed a larger size than other lipids.<sup>16</sup>



**Figure 2.8.** Mean particle size (A, C) and surface potential (B, D) of the lipoplexes at different N/P ratios. The lipids of C12 (A, B) and C16 (C, D) chain lengths were used to measure the particle size and surface potential of the corresponding lipoplexes. DLS measurements were performed at room temperature. The molar ratio of synthetic lipid and PE was 1:1.

## 2.2.8. Cell viability and *in vitro* Gene Transfection Efficiency

### 2.2.8.1 Cell Viability

The cytotoxicity of the lipids was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using human embryonic kidney 293 (HEK-293T) and baby hamster kidney fibroblasts (BHK-21) cell lines.<sup>23</sup> The lipids **2.16a**, **2.17a**, and **2.19a** were selected for investigating their applicability because

of their higher DNA-binding abilities. The MTT results showed that the IC<sub>50</sub> values of the lipids were within 106–165 and 80–144  $\mu$ M for HEK-293T and BHK-21 cell lines, respectively (Table 2.2). Overall, the IC<sub>50</sub> values indicate the low toxicity of these lipids, which is useful for their successful application as a nonviral gene delivery system.<sup>7a-b, 16</sup>

**Table 2.2.** The IC<sub>50</sub> values of the lipids in HEK-293T and BHK-21 cell lines

| Lipid        | Cell line | IC <sub>50</sub> ( $\mu$ M) |
|--------------|-----------|-----------------------------|
| <b>2.16a</b> | HEK-293T  | 106.52 $\pm$ 5.34           |
|              | BHK-21    | 109.54 $\pm$ 5.76           |
| <b>2.17a</b> | HEK-293T  | 107.22 $\pm$ 8.54           |
|              | BHK-21    | 144.34 $\pm$ 9.23           |
| <b>2.19a</b> | HEK-293T  | 165.39 $\pm$ 7.34           |
|              | BHK-21    | 79.89 $\pm$ 8.45            |

### 2.2.8.2 Gene Transfection Efficiency of the Lipoplexes

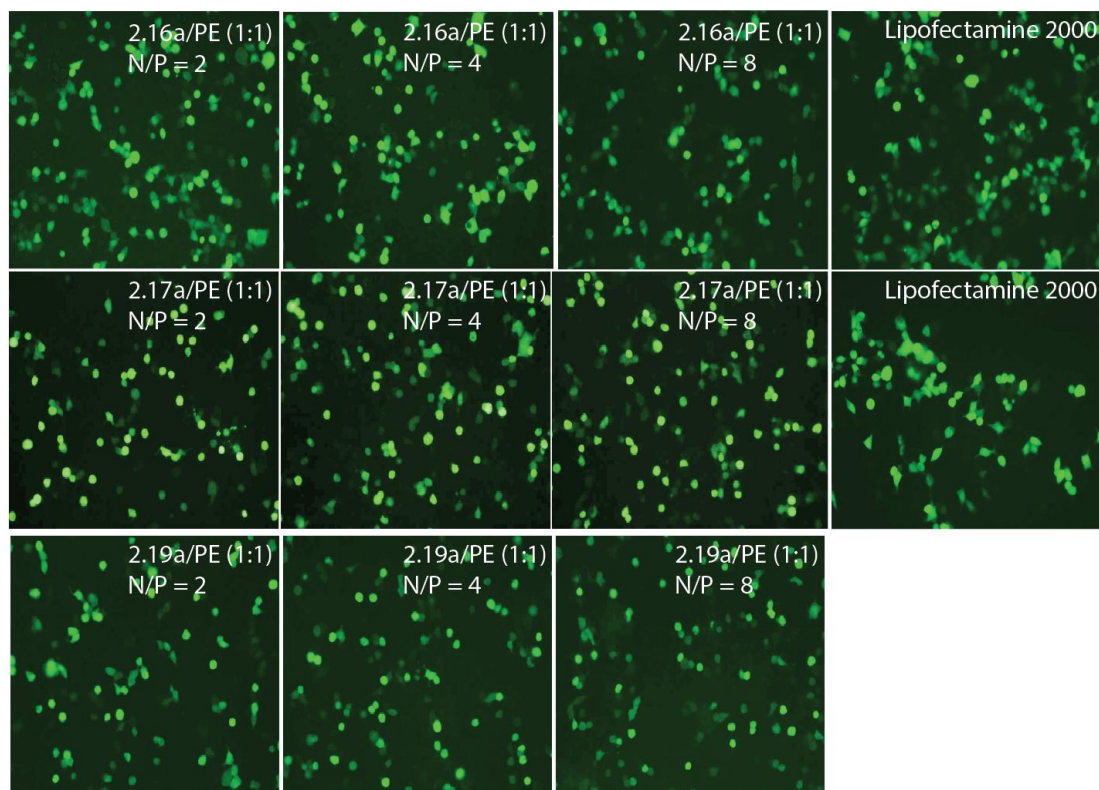
The outcome of the gel retardation assay and the formation of stable lipoplex suggest that the synthesized cationic lipids can potentially deliver the genetic material to cells. Hence, in vitro gene transfection efficiencies of the lipids were investigated by fluorescence microscopy assay in HEK-293T cells using pMMP7-GFP plasmid DNA encoding the GFP-tagged MMP7 enzyme. It is well documented that fusogenic lipid-like PE is usually required for effective transfection by the cationic lipids.<sup>23</sup> The synthetic lipids **2.16a**, **2.17a**, and **2.19a** showed strong DNA-binding abilities (with N/P ratio >2) among all of the tested lipids. In this regard, the cellular assays were performed using only these potent lipids. The liposomes were prepared in combination with co-lipid PE (1:1 mole ratio). The liposomes with helper lipid-like PE show higher transfection and lower cytotoxicity than those without PE.<sup>32</sup> The lipoplexes were prepared at an N/P ratio of 2–8 using pMMP7-GFP DNA as the reporter gene, and Lipofectamine 2000 was used as the positive control (Figures 2.9). The cellular expression of GFP protein was monitored to measure the transfection efficiency of the lipids. The outcome of the gene transfection experiment revealed that lipids **2.16a**, **2.17a**, and **2.19a** (at an N/P ratio of 1–4) had a

comparable transfection efficiency to the commercially available Lipofectamine 2000 (Figures 2.9). The lipids with C16 chain lengths showed comparatively weak DNA-binding efficiencies. Hence, the transfection efficacies of those lipids were not investigated. The presence of unsaturated hydrophobic tails causes weak intermolecular hydrophobic interactions, leading to the formation of less stable liposomes, which generally do not have any significant effect on the DNA binding and delivery efficacies; hence, those classes of lipids were also not investigated. The final concentrations of the lipids used for the delivery of pMMP7-GFP DNA were within 15–120 nM (for N/P ratios 1–8), which are much lower than their IC<sub>50</sub> values (106–165  $\mu$ M; obtained from the MTT assay). Hence, the toxicity of these lipids could have a negligible impact on their gene transfection efficiencies. We also evaluated the cellular uptake of pMMP7-GFP in HEK-293T cells at different N/P ratios. The internalization of the lipoplexes significantly influences the transfection efficiency of the lipids.

We performed the flow cytometry assay to investigate the cellular uptake of the DNA.<sup>17</sup> After transfection, the percentage of cells positive for GFP-labeled pMMP7 was calculated and is shown in (Figure 2.46). For fluorescence-activated cell sorting (FACS) analyses, experiments were performed using the FL1 channel for green fluorescence, and the data were analyzed using FCS Express 6 Flow Cytometry software. The autofluorescence was detected using nontransfected cells as control indicated as a peak in the histogram plot using the FL1 channel. The M1 region is indicated in plots capturing green fluorescence intensity in transfected cells, which is more than autofluorescence intensity. The percentages of GFP-positive cells were calculated to measure the cellular uptake properties of the lipids. The outcome of the flow cytometry analysis showed that there was only a little difference between **2.16a**, **2.19a** (N/P = 2 and 4), and Lipofectamine 2000, indicating that the lipoplexes of these synthetic lipids have the comparable intracellular delivery ability. The cellular uptake efficiencies of lipids **2.16a** and **2.19a** were higher than that of **2.17a**, which is in good agreement with their DNA-binding and transfection efficiencies. However, the transfection efficiencies of all of the tested lipids decrease with N/P ratio >4. A similar consequence was observed for their DNA transfection efficiencies, suggesting that very high positive surface potentials limit their transfection abilities in healthy HEK-293T cells. The outer membrane of the normal healthy cells is



generally zwitterionic because of the high abundance of zwitterionic lipids like phosphatidylcholine and others.<sup>33</sup> We are currently performing comprehensive transfection mechanism studies of such types of lipids in different cell lines.



**Figure 2.9.** Fluorescence microscopy images of the MMP7-GFP expression in HEK-293T cells by lipid **2.16a**, **2.17a**, and **2.19a** at various N/P ratios. Scale bar = 100  $\mu$ M.

### 2.3. Summary

The sunlight-mediated synthetic reaction conditions in the aqueous medium have been developed to synthesize a series of small molecules and lipids. The thiol-yne/ene click reaction was performed in the aqueous medium in the presence of a catalytic amount of tert-butyl hydroperoxide (TBHP) with a very high hydrothiolation efficiency. The biophysical properties of the lipids, such as particle size, surface potential, phase-transition temperature, and DNA-binding ability, were thoroughly investigated. Most importantly, all of these phosphorous-free lipids form stable vesicles, but only the cationic lipids form stable lipoplexes. The structure-based DNA-binding studies of the lipids revealed that the presence of the  $\beta$ -hydroxy group in the linker region plays a vital role in forming stable lipoplexes. Additional



studies also revealed that the hydrophobic chain length significantly affects the DNA-binding abilities of the lipids, and C12 was found to be more suitable than C16 chain length. The potent cationic lipids displayed much lower cytotoxicity. Interestingly, these cationic lipids also showed comparable gene transfection and cellular uptake properties with the commercially available Lipofectamine 2000, signifying their prospective to be favorable nonviral gene vectors. Therefore, the design and synthesis of such sunlight-driven cationic lipids may be a promising approach for lipid-based gene delivery systems. However, further optimization of the lipid structure to improve the gene delivery activities is required to establish their *in vivo* applications. Overall, such mild reaction conditions can be applied for the smart design of new or improvement of existing lipid-based gene delivery systems. This synthetic strategy can also be used to synthesize other small or macromolecules for pharmaceutical materials and others in the aqueous medium.

## 2.4. Experimental section

### 2.4.1. Synthesis of the compounds

#### 2.4.1.1. General information

All reagents were purchased from Sigma-Aldrich, Merck, Himedia, and used directly without further purification. Column chromatography was performed using 60–120 mesh silica gels. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F<sub>254</sub> (0.25 mm). <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded at 400 and 100 MHz, respectively, with a Varian AS400 spectrometer and 600 and 151 MHz with Bruker spectrometers, using tetramethylsilane (TMS) as an internal standard with CDCl<sub>3</sub>, dimethyl sulfoxide (DMSO)-*d*<sub>6</sub>. The coupling constants (*J* values) and chemical shifts ( $\delta_{\text{ppm}}$ ) were reported in hertz (Hz) and parts per million (ppm), respectively, downfield from tetramethylsilane using residual chloroform ( $\delta$  = 7.24 for <sup>1</sup>H NMR,  $\delta$  = 77.23 ppm for <sup>13</sup>C NMR) as an internal standard. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broadened). High-resolution mass spectroscopy (HRMS) images were recorded on an Agilent Q-TOF mass spectrometer with Z-spray source using built-in software for analysis of the recorded data. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PE) was purchased from Avanti Polar Lipids (Alabaster, AL). Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers.

### 2.4.1.2. General Procedure for the Thiol–Ene/Yne Click Reaction

All of the compounds were synthesized using the optimized reaction condition (Table 1, entry 17). To a solution of alkynyl/alkenyl derivative (0.46 mmol) in water (2 mL) or H<sub>2</sub>O/DMF (10:0.5) solution mixture (2 mL) in a glass vial were added thiol derivative (1.0 mmol) and TBHP (0.023 mmol) solution and vortexed for 2 min. The reaction vial was then exposed to sunlight (weather condition: sunny, temperature, 30–36 °C) and kept for 30 min. After completion of the reaction, the mixture was diluted with cold water and ethyl acetate. The organic layer was extracted and washed with brine and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The organic solvent was removed under reduced pressure. The reaction mixture was purified by column chromatography using EtOAc/hexane (2–40%) solvent gradient.

### 2.4.1.3. General procedure for the synthesis of targeted compounds

#### 2.4.1.3.1. Synthesis of 2,3-bis(dodecylthio)propan-1-ol (**2.3a**)

Following the general procedure (in water), using propargyl alcohol (40 mg, 0.71 mmol) and dodecanthiol (318 mg, 1.57 mmol) provided 300 mg (94% yield, time = 30 min) of **2.3a** as a colorless liquid; <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 3.81–3.78 (m, 1H), 3.67–3.64 (m, 1H), 2.91–2.87 (m, 1H), 2.82–2.79 (m, 1H), 2.71–2.67 (m, 1H), 2.57–2.51 (m, 4H), 1.59–1.54 (m, 4H), 1.39–1.32 (m, 4H), 1.30–1.24 (m, 33H), 0.86 (t, 3H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 63.3, 48.8, 35.0, 33.1, 32.0, 31.2, 30.1, 29.8, 29.7, 29.6, 29.5, 29.3, 29.0, 29.0, 22.8, 14.20; HRMS [electrospray ionization (ESI)] calcd for C<sub>27</sub>H<sub>56</sub>O<sub>2</sub>S [M + K]<sup>+</sup>: 499.3614, found: 499.3622.

#### 2.4.1.3.2. Synthesis of 2,3-bis(dodecylthio)propan-1-amine (**2.3b**)

Following the general procedure (in water), using propargylamine (40 mg, 0.72 mmol) and dodecanthiol (324 mg, 1.59 mmol) provided 305 mg (92% yield, time = 30 min) of **2.3b** as a yellowish semiliquid; <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 3.03–3.00 (m, 1H), 2.82–2.81 (m, 3H), 2.66–2.62 (m, 1H), 2.55–2.48 (m, 4H), 1.58–1.53 (m, 4H), 1.36–1.32 (m, 4H), 1.28–1.23 (m, 32H), 0.85 (t, 6H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 49.6, 44.3, 35.9, 33.2, 32.0, 31.1, 30.1, 29.8, 29.8, 29.7, 29.6, 29.5, 29.4, 29.1, 29.0, 22.8, 14.2; HRMS (ESI) calcd for C<sub>27</sub>H<sub>57</sub>NS<sub>2</sub> [M + H]<sup>+</sup>: 460.4005, found: 460.4005.

**2.4.1.3.3. Synthesis of 2,3-bis(dodecylthio)butane-1,4-diol (2.3c)**

Following the general procedure (in water), using but-2-yne-1,4-diol (40 mg, 0.46 mmol) and dodecanthiol (207.1 mg, 1.02 mmol) provided 201 mg (89% yield, time = 30 min) of **2.3c** as a colorless liquid;  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  3.88–3.74 (m, 2H), 3.06–2.84 (m, 4H), 2.68–2.60 (m, 2H), 2.58–2.51 (m, 2H), 1.68–1.47 (m, 4H), 1.37–1.31 (m, 4H), 1.29–1.25 (m, 36H), 0.87 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  62.3, 51.5, 32.7, 31.9, 30.0, 29.6, 29.6, 29.6, 29.5, 29.3, 29.2, 29.2, 28.8, 22.6, 14.1; HRMS (ESI) calcd for  $\text{C}_{28}\text{H}_{58}\text{O}_2\text{S}_2$   $[\text{M} + \text{Na}]^+$ : 513.3770, found: 513.3770.

**2.4.1.3.4. Synthesis of dodecyl(1-phenylvinyl)sulfane (2.3d)**

Following the general procedure [in  $\text{H}_2\text{O}$ /DMF solvent mixture (1:0.01; v/v)], using ethynylbenzene (40 mg, 0.39 mmol) and dodecanthiol (174.56 mg, 0.86 mmol) provided 102 mg (85% yield, time = 30 min) of **2.3d** as a colorless liquid;  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  7.47–7.46 (d, 1H), 7.34–7.31 (m, 1H), 7.25–7.22 (m, 1H), 7.18–7.14 (m, 2H), 6.70 (d,  $J = 18$  Hz, 1H), 6.43 (d,  $J = 18$  Hz, 1H), 2.78–2.74 (m, 2H), 1.69–1.64 (m, 2H), 1.43–1.36 (m, 2H), 1.31–1.24 (m, 18H), 0.87 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  137.3, 128.7, 128.3, 127.9, 126.9, 126.7, 125.6, 125.5, 36.1, 32.8, 32.1, 30.4, 29.8, 29.8, 29.7, 29.6, 29.5, 29.4, 29.0, 28.8, 22.9, 14.3; HRMS (ESI) calcd for  $\text{C}_{20}\text{H}_{32}\text{S}$   $[\text{M} + \text{H}]^+$ : 305.2297, found: 305.2305.

**2.4.1.3.5. Synthesis of 2,3-bis(dodecylthio)-3-phenylpropan-1-ol (2.3e)**

Following the general procedure [in  $\text{H}_2\text{O}$ /DMF solvent mixture (1:0.01; v/v)], using 3-phenylprop-2-yn-1-ol (40 mg, 0.30 mmol) and dodecanthiol (135 mg, 0.66 mmol) provided 91 mg (90% yield, time = 30 min) of **2.3e** as a colorless liquid;  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  7.63–7.59 (m, 2H), 7.36–7.32 (m, 2H), 7.26–7.22 (m, 1H), 6.82 (s, 1H), 4.34 (s, 4H), 2.77–2.66 (m, 2H), 1.64–1.50 (m, 2H), 1.33–1.29 (m, 2H), 1.25–1.22 (m, 17H), 0.88 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  136.2, 135.6, 129.8, 129.6, 129.4, 128.0, 127.3, 66.9, 31.9, 31.3, 29.8, 29.6, 29.5, 29.4, 29.3, 29.1, 28.7, 22.6, 14.1; HRMS (ESI) calcd for  $\text{C}_{21}\text{H}_{34}\text{OS}$   $[\text{M} + \text{H}]^+$ : 335.2403, found: 335.2416.

**2.4.1.3.6. Synthesis of 2-((2-hydroxyethyl)thio)-3-phenylprop-2-en-1-ol (2.3f)**

Following the general procedure [in  $\text{H}_2\text{O}$ /DMF solvent mixture (1:0.01; v/v)], using 3-phenylprop-2-yn-1-ol (40 mg, 0.30 mmol) and 2-mercaptoethanol (28.4 mg, 0.36

mmol) provided 58 mg (92% yield, time = 30 min) of **2.3f** as a yellowish semiliquid;  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  3.27–2.82 (m, 2H), 2.77–2.64 (m, 1H), 1.87–1.60 (m, 2H), 1.49–1.31 (m, 3H), 1.34–1.25 (m, 17H), 0.89 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  135.8, 134.3, 132.0, 129.3, 128.4, 128.2, 127.6, 127.4, 67.4, 61.2, 34.7; HRMS (ESI) calcd for  $\text{C}_{11}\text{H}_{14}\text{O}_2\text{S}$   $[\text{M} + \text{Na}]^+$ : 233.0607, found: 233.0607.

#### 2.4.1.3.7. Synthesis of 3-(dodecylthio)-2-methylpropanoic Acid (2.5a)

Following the general procedure [in  $\text{H}_2\text{O}$ /DMF solvent mixture (1:0.01; v/v)], using methacrylic acid (40 mg, 0.46 mmol) and dodecanthiol (112.9 mg, 0.55 mmol) provided 119 mg (90% yield, time = 30 min) of **2.5a** as a white solid;  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  3.27–2.82 (m, 2H), 2.77–2.64 (m, 1H), 1.87–1.60 (m, 2H), 1.49–1.31 (m, 3H), 1.34–1.25 (m, 17H), 0.89 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$ ; HRMS (ESI) calcd for  $\text{C}_{16}\text{H}_{32}\text{O}_2\text{S}$   $[\text{M} + \text{Na}]^+$ : 311.2015, found: 311.2016.

#### 2.4.1.3.8. Synthesis of 2-(dodecylthio)butane-1,4-diol (2.5b)

Following the general procedure [in  $\text{H}_2\text{O}$ /DMF solvent mixture (1:0.01; v/v)], using but-2-ene-1,4-diol (40 mg, 0.45 mmol) and dodecanthiol (110 mg, 0.54 mmol) provided 114 mg (87% yield, time = 30 min) of **2.5b** as a white solid;  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  4.09–4.03 (m, 1H), 3.87–3.77 (m, 1H), 3.08–3.05 (m, 1H), 3.01–2.73 (m, 1H), 2.32–2.19 (m, 1H), 2.00–1.92 (m, 1H), 1.88–1.59 (m, 2H), 1.52–1.39 (m, 1H), 1.32–1.25 (m, 16H), 0.87 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  61.5, 60.0, 52.2, 31.9, 29.7, 29.6, 29.5, 29.3, 29.2, 29.0, 28.8, 28.5, 27.6, 22.9, 22.6, 21.2, 14.0; HRMS (ESI) calcd for  $\text{C}_{16}\text{H}_{34}\text{O}_2\text{S}$   $[\text{M} + \text{Na}]^+$ : 313.2172, found: 313.2172.

#### 2.4.1.3.9. Synthesis of dodecyl(phenethyl)sulfane (2.5c)

Following the general procedure [in  $\text{H}_2\text{O}$ /DMF solvent mixture (1:0.01; v/v)], using styrene (40 mg, 0.38 mmol) and dodecanthiol (93 mg, 0.46 mmol) provided 99 mg (85% yield, time = 30 min) of **2.5c** as a colorless liquid;  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  7.31–7.29 (m, 2H), 7.23–7.20 (m, 3H), 2.90–2.87 (m, 2H), 2.78–2.76 (m, 2H), 2.54–2.52 (m, 2H), 1.59–1.57 (m, 2H), 1.39–1.34 (m, 2H), 1.31–1.26 (m, 17H), 0.88 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  140.9, 128.6, 126.5, 36.6, 33.8, 32.5, 32.1, 29.8, 29.8, 29.8, 29.7, 29.5, 29.4, 29.1, 22.9, 14.3; HRMS (ESI) calcd for  $\text{C}_{20}\text{H}_{34}\text{S}$   $[\text{M} + \text{H}]^+$ : 307.2454, found: 307.2457.

**2.4.1.3.10. Synthesis of dodecyl(4-methylphenethyl)sulfane (2.5d)**

Following the general procedure [in H<sub>2</sub>O/DMF solvent mixture (1:0.01; v/v)], using 1-methyl-4-vinylbenzene (40 mg, 0.33 mmol) and dodecanthiol (82 mg, 0.40 mmol) provided 89 mg (85% yield, time = 30 min) of **2.5d** as a colorless semiliquid; <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 7.14–7.11 (m, 4H), 2.88–2.85 (m, 2H), 2.78–2.76 (m, 2H), 2.56–2.53 (m, 2H), 2.34 (s, 3H), 1.63–1.58 (m, 2H), 1.47–1.32 (m, 2H), 1.35–1.29 (m, 18H), 0.91 (t, 3H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 137.7, 135.7, 129.1, 128.3, 36, 33.8, 33.2, 31.9, 29.7, 29.7, 29.6, 29.6, 29.5, 29.3, 29.2, 28.9, 22.7, 21.0, 14.1; HRMS (ESI) calcd for C<sub>21</sub>H<sub>36</sub>S [M + H]<sup>+</sup>: 321.2610, found: 321.2612.

**2.4.1.3.11. Synthesis of 2-((4-methylphenethyl)thio)ethanol (2.5e)**

Following the general procedure [in H<sub>2</sub>O/DMF solvent mixture (1:0.01; v/v)], using ethynylbenzene (40 mg, 0.39 mmol) and 2-mercaptoethanol (35.98 mg, 0.46 mmol) provided 61 mg (87% yield, time = 30 min) of **2.5e** as a light yellowish semiliquid; <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 7.34–7.31 (m, 2H), 7.24–7.20 (m, 3H), 3.71 (t, 2H), 2.91–2.89 (m, 2H), 2.81–2.78 (m, 2H), 2.73 (t, 2H), 2.26 (s, 1H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 140.2, 128.5, 128.5, 126.6, 60.3, 36.3, 35.5, 33.2; HRMS (ESI) calcd for C<sub>10</sub>H<sub>14</sub>OS [M + K]<sup>+</sup>: 221.0569, found: 221.0591.

**2.4.1.3.12. Synthesis of 3-((4-methylphenethyl)thio)propanoic Acid (2.5f)**

Following the general procedure [in H<sub>2</sub>O/DMF solvent mixture (1:0.01; v/v)], using 1-methyl-4-vinylbenzene (40 mg, 0.33 mmol) and 3-mercaptopropanoic acid (42 mg, 0.39 mmol) provided 66 mg (89% yield, time = 30 min) of **2.5f** as a white solid; <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 3.27–2.82 (m, 2H), 2.77–2.64 (m, 1H), 1.87–1.60 (m, 2H), 1.49–1.39 (m, 3H), 1.34–1.25 (m, 17H), 0.89 (t, 3H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 177.9, 137.2, 135.9, 129.1, 128.3, 35.7, 35.6, 35.5, 33.7, 26.9, 21.0, 19.6; HRMS (ESI) calcd for C<sub>12</sub>H<sub>16</sub>O<sub>2</sub>S [M + H]<sup>+</sup>: 225.0944, found: 225.0942.

**2.4.1.3.13. Synthesis of (1S)-((1S,2S,4S)-5-(2-(dodecylthio)ethyl)quinuclidin-2-yl)(6-methoxyquinolin-4-yl)methanol (2.5g)**

Following the general procedure [in H<sub>2</sub>O/DMF solvent mixture (1:0.01; v/v)], using quinine (40 mg, 0.12 mmol) and dodecanthiol (30 mg, 0.15 mmol) provided 56 mg (87% yield, time = 30 min) of **2.5g** as a white solid; <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>)

$\delta_{\text{ppm}}$  8.68–8.66 (m, 1H), 7.93–7.91 (m, 1H), 7.52–7.49 (m, 2H), 7.40–7.37 (m, 1H), 5.28–5.27 (d, 1H), 3.90 (s, 3H), 3.28–3.18 (m, 1H), 3.07–3.01 (m, 1H), 2.88–2.82 (m, 1H), 2.44–2.37 (m, 4H), 1.81 (s, 2H), 1.71–1.63 (m, 4H), 1.55–1.52 (m, 2H), 1.46–1.44 (m, 2H), 1.23–1.21 (m, 21H), 0.85–0.81 (m, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  157.2, 149.7, 147.9, 144.3, 131.5, 127.4, 121.4, 119.5, 102.9, 71.2, 60.8, 57.7, 56.2, 55.9, 52.0, 42.3, 34.8, 31.7, 31.5, 29.7, 29.5, 29.4, 29.3, 29.3, 29.1, 29.0, 28.9, 28.5, 28.4, 25.7, 25.5, 23.9, 23.1, 22.5, 14.3; HRMS (ESI) calcd for  $\text{C}_{32}\text{H}_{50}\text{N}_2\text{O}_2\text{S}$   $[\text{M} + \text{H}]^+$ : 527.3666, found: 527.3666.

#### 2.4.1.3.14. Synthesis of 1-(dimethylamino)-3-(prop-2-yloxy)propan-2-ol (2.7)

To a stirring solution of 2-((prop-2-yn-1-yloxy)methyl)oxirane (**6**) (8.92 mmol) in dry dichloromethane solvent dimethylamine (26.78 mmol) was added, and the resulting mixture was stirred for 12 h at room temperature under  $\text{N}_2$  atmosphere. The progress of the reaction was monitored by TLC. After maximum consumption of compound **6**, the solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel using a gradient solvent system of dichloromethane and methanol (10:0.6) to afford compound **2.7** as a yellow liquid. Characterization of compound **7**: yellow liquid (yield 73%);  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  4.15 (d,  $J = 2.5$  Hz, 2H), 3.92–3.86 (m, 1H), 3.84 (m, 1H), 3.54–3.51 (m, 1H), 3.45 (dd,  $J = 9.8, 5.7$  Hz, 1H), 2.42 (m, 2H), 2.25 (m, 6H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  79.5, 74.7, 72.3, 66.6, 61.9, 58.6, 45.5; HRMS (ESI) calcd for  $\text{C}_8\text{H}_{15}\text{NO}_2$   $[\text{M} + \text{H}]^+$ : 158.1136, found: 158.1126.

#### 2.4.1.3.15. Synthesis of 2-hydroxy-*N,N,N*-trimethyl-3-(prop-2-yn-1-yloxy)propan-1-aminium (2.8)

To a stirring solution of 1-(dimethylamino)-3-(prop-2-yloxy)propan-2-ol (**7**, 0.827 mmol) in acetonitrile (10 mL) was added  $\text{K}_2\text{CO}_3$  (0.827 mmol), and the reaction mixture was continued to stir for 30 min at room temperature. After that, iodomethane (0.82 mmol) was added, and the reaction mixture was heated under reflux condition for 12 h. After maximum consumption of compound **2.7**, the reaction mixture was cooled down to room temperature and unused  $\text{K}_2\text{CO}_3$  was removed by filtration. Removal of organic solvent under reduced pressure yielded an oily crude product. The addition of diethyl ether to this oily crude product yielded the target



product as a colorless crystalline solid, which was used without further purification. Characterization of compound **2.8**: colorless solid (yielded 87%);  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta_{\text{ppm}}$  4.25 (m, 1H), 4.20–4.19 (m, 2H), 3.50 (t,  $J = 2.4$  Hz, 1H), 3.48–3.46 (m, 1H), 3.39–3.33 (m, 3H), 3.15 (s, 9H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )  $\delta_{\text{ppm}}$  80.5, 78.1, 72.3, 68.5, 64.4, 58.4, 53.0; HRMS (ESI) calcd for  $\text{C}_9\text{H}_{18}\text{NO}_2$   $[\text{M}]^+$ : 172.1332, found: 172.1341.

#### 2.4.1.3.16. Synthesis of *N*-(carboxymethyl)-2-hydroxy-*N,N*-dimethyl-3-(prop-2-yn-1-yloxy)propan-1-aminium (**2.9**)

To a stirring solution of 1-(dimethylamino)-3-(prop-2-yloxy)propan-2-ol (**2.7**, 1.27 mmol) in dichloromethane (10 mL) was slowly added (dropwise) a solution of bromoacetic acid (3.82 mmol) in dichloromethane (2 mL) at room temperature under  $\text{N}_2$  atmosphere. The resulting reaction mixture was stirred for 48 h; after that, a gummy part was observed below the reaction solution. Removal of organic solvent under reduced pressure yielded a gummy mass, which was washed with diethyl ether several times. The solvent (diethyl ether) wash yielded the target product, which was used without further purification. Characterization of compound **2.9**: light yellow solid (yield 85%);  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  4.22–4.17 (m, 1H), 4.16 (d,  $J = 2.3$  Hz, 2H), 3.74–3.73 (m, 2H), 3.66–3.62 (m, 1H), 3.56–3.52 (m, 1H), 3.46–3.42 (m, 2H), 3.34–3.30 (m, 1H), 3.23–3.20 (m, 6H);  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  170.8, 85.2, 82.8, 77.1, 70.6, 69.1, 63.1, 56.5, 56.3; HRMS (ESI) calcd for  $\text{C}_{10}\text{H}_{18}\text{NO}_4$   $[\text{M}]^+$ : 216.1230, found: 216.1231.

#### 2.4.1.3.17. Synthesis of 2,2'-((2-hydroxy-3-(prop-2-yn-1-yloxy)propyl)azanediyl)bis(ethan-1-ol) (**2.10**)

To a stirring solution of compound **2.6** (4.42 mmol) in dichloromethane (10 mL) was added a solution of diethanolamine (8.84 mmol) in dichloromethane (2 mL) at room temperature under  $\text{N}_2$  atmosphere. The resulting reaction mixture was stirred for 8 h. After completion of the reaction, the unused solvent was removed under reduced pressure. The yellowish crude reaction mixture was purified by column chromatography on silica gel using a gradient solvent system of dichloromethane and methanol (10:1.2). Characterization of compound **2.10**: light yellow viscous liquid (yield 82%);  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  4.82 (brs, 3H), 4.17–4.16 (m, 2H),

3.93–3.88 (m, 1H), 3.70–3.66 (m, 2H), 3.53–3.45 (m, 4H), 2.75–2.70 (m, 2H), 2.59–2.54 (m, 1H), 2.44–2.37 (m, 4H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  79.5, 74.8, 72.1, 67.5, 59.3, 58.6, 58.3, 57.3; HRMS (ESI) calcd for  $\text{C}_8\text{H}_{15}\text{NO}_2$   $[\text{M} + \text{H}]^+$ : 232.1543, found: 232.1539.

#### 2.4.1.3.18. Synthesis of 2-hydroxy-*N,N*-bis(2-hydroxyethyl)-*N*-methyl-3-(prop-2-yn-1-yloxy)propan-1-aminium (2.11)

To a stirring solution of 2,2'-((2-hydroxy-3-(prop-2-yn-1-yloxy)propyl)azanediyl)bis(ethan-1-ol) (10, 0.92 mmol) in acetonitrile (10 mL) was added and  $\text{K}_2\text{CO}_3$  (0.80 mmol), and the reaction mixture was continued to stir for 30 min at room temperature under  $\text{N}_2$  atmosphere. After that, iodomethane (0.92 mmol) was added, and the reaction mixture was heated under reflux condition for 12 h. After maximum consumption of compound **2.5**, the reaction mixture was cooled down to room temperature, and the unused  $\text{K}_2\text{CO}_3$  was removed by filtration. Removal of organic solvent under reduced pressure yielded a viscous residue. The residue was purified by column chromatography on silica gel using a gradient solvent system of dichloromethane, methanol, and water (5:1:0.1). Characterization of compound **2.11**: White solid (yield 88%);  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta_{\text{ppm}}$  4.26 (m, 1H), 4.19 (s, 1H), 3.84 (m, 2H), 3.59 (m, 1H), 3.57 (m, 4H), 3.54 (m, 2H), 3.44 (m, 4H), 3.37 (m, 1H), 3.16 (s, 3H);  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO}-d_6$ )  $\delta_{\text{ppm}}$  80.4, 78.1, 72.3, 65.7, 64.9, 64.2, 58.4, 55.4, 50.5; HRMS (ESI) calcd for  $\text{C}_{11}\text{H}_{22}\text{NO}_4$   $[\text{M}]^+$ : 232.1549, found: 232.1554.

#### 2.4.1.3.19. Synthesis of *tert*-butyl (2-((2-hydroxy-3-(prop-2-yn-1-yloxy)propyl)amino)ethyl)carbamate (2.12)

To a stirring solution of compound **2.6** (1.76 mmol) in dichloromethane (5 mL) was added a solution of *tert*-butyl (2-aminoethyl)carbamate (3.53 mmol) in dichloromethane at 0 °C under  $\text{N}_2$  atmosphere. Then, the reaction mixture was warmed up to room temperature and allowed to stir for 8 h at room temperature. After completion of the reaction, the remaining solvent was removed under reduced pressure. The crude reaction mixture was purified by column chromatography on silica gel using a gradient solvent system of dichloromethane and methanol (10:0.6) to afford compound **2.12** as a yellow liquid. Characterization of compound **2.12**:

yellow liquid (yield 79%)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  5.33–5.32 (m, 1H), 4.16 (s, 2H), 3.91–3.88 (m, 1H), 3.55–3.47 (m, 2H), 3.26–3.17 (m, 4H), 2.74–2.72 (m, 2H), 2.65 (t,  $J = 10.3$  Hz, 1H), 2.45 (s, 1H), 1.40 (s, 9H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  156.1, 79.3, 79.1, 74.7, 72.4, 68.4, 58.4, 51.5, 49.0, 39.8, 28.3; HRMS (ESI) calcd for  $\text{C}_{13}\text{H}_{24}\text{N}_2\text{O}_4$   $[\text{M} + \text{H}]^+$ : 273.1809, found: 273.1827.

#### 2.4.1.3.20. Synthesis of *N*-(2-((*tert*-butoxycarbonyl)amino)ethyl)-2-hydroxy-*N,N*-dimethyl-3-(prop-2-yn-1-yloxy)propan-1-aminium (2.13)

To a stirring solution of *tert*-butyl (2-((2-hydroxy-3-(prop-2-yn-1-yloxy)propyl)amino)ethyl)carbamate (12, 0.55 mmol) in acetonitrile (10 mL) was added  $\text{K}_2\text{CO}_3$  (0.55 mmol), and the reaction mixture was continued to stir for 30 min at room temperature under  $\text{N}_2$  atmosphere. After that, iodomethane (1.10 mmol) was added and the reaction mixture was heated under reflux condition for 12 h. After maximum consumption of compound **2.12**, the reaction mixture was cooled down to room temperature, and unused  $\text{K}_2\text{CO}_3$  was removed by filtration. The removal of organic solvent under reduced pressure yielded a viscous residue. The crude reaction mixture was purified by column chromatography on silica gel using a gradient solvent system of dichloromethane and methanol (10:1) to afford compound **2.13** as a brown sticky liquid. Characterization of compound **2.13**: brown gummy liquid (yield 92%)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  5.85–5.83 (m, 1H), 5.30 (s, 1H), 4.62–4.61 (m, 1H), 4.44 (s, 1H), 4.22 (s, 2H), 3.83–3.81 (m, 2H), 3.69 (m, 4H), 3.45–3.43 (m, 6H), 2.60 (s, 1H), 2.38–2.33 (m, 1H), 1.42 (s, 9H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  156.2, 80.4, 79.1, 75.9, 71.4, 67.0, 64.6, 64.3, 58.8, 53.5, 35.2, 28.4; HRMS (ESI) calcd for  $\text{C}_{15}\text{H}_{29}\text{N}_2\text{O}_4$   $[\text{M}]^+$ : 301.2127, found: 301.2133.

#### 2.4.1.3.21. Synthesis of *N*-(2-aminoethyl)-2-hydroxy-*N,N*-dimethyl-3-(prop-2-yn-1-yloxy)propan-1-aminium (2.14)

To a stirring solution of compound **2.13** (0.465 mmol) in dichloromethane (9 mL) was added trifluoroacetyl (TFA) (1 mL; dropwise addition) at 0 °C and stirring was continued for 30 min. After that, the reaction mixture was warmed up to room temperature, and stirring was continued for another 3 h. After the consumption of the maximum starting material, the solvent was removed under reduced pressure. The oily liquid was then washed with diethyl ether ( $3 \times 5$  mL). The excess TFA was

removed by purging the N<sub>2</sub> gas through the oily liquid yielded as a brown sticky liquid, which was used without further purification. Characterization of compound **2.14**: brown gummy solid (yield 82%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ<sub>ppm</sub> 4.28–4.23 (m, 1H), 4.19 (brs, 2H), 4.12–3.95 (m, 2H), 3.65 (t, *J* = 7.7 Hz, 2H), 3.49–3.47 (m, 2H), 3.44–3.43 (m, 2H), 3.40–3.36 (m, 1H), 3.44–3.25 (m, 1H) 3.19 (m, 6H); <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ<sub>ppm</sub> 80.3, 78.1, 72.0, 66.9, 64.3, 60.6, 58.4, 52.3, 39.9, 32.9; HRMS (ESI) calcd for C<sub>10</sub>H<sub>21</sub>N<sub>2</sub>O<sub>2</sub> [M]<sup>+</sup>: 201.1603, found: 201.1617.

#### 2.4.1.3.22. General Synthesis of Lipid Molecules (2.15a – 2.20a)

All lipids were synthesized using the optimized reaction conditions. The H<sub>2</sub>O/DMF [1:0.01 (v/v)] was added to a vial containing alkynyl derivative (0.46 mmol), dodecanethiol/hexadecanethiol (1.0 mmol), and TBHP (0.023 mmol) solution and vortexed for 2 min. The vial was sealed and exposed to sunlight (weather condition: sunny; temperature, 30–36 °C). After 30 min, the reaction mixture was diluted with cold water and ethyl acetate. The organic layer was extracted and washed with brine and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The organic solvent was removed under reduced pressure. The reaction mixture was purified by column chromatography using MeOH/CH<sub>2</sub>Cl<sub>2</sub> (2–10%) solvent gradient to afford the corresponding lipid.

#### 2.4.1.3.23. Characterization of Lipid 2.15a

Yield 82%. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 4.01–3.96 (m, 4H), 3.7–3.70 (m, 1H), 3.68–3.63 (m, 1H), 3.54–3.45 (m, 2H), 2.97–2.93 (m, 1H), 2.85–2.76 (m, 2H), 2.60–2.52 (m, 4H), 2.40 (s, 6H), 1.59–1.54 (m, 4H), 1.38–1.32 (m, 4H), 1.29–1.24 (m, 37H), 0.87 (t, *J* = 6.9 Hz, 6H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 73.5, 73.4, 73.1, 73.1, 66.4, 61.8, 61.7, 45.6, 45.4, 34.9, 33.3, 31.9, 31.7, 29.9, 29.7, 29.7, 29.7, 29.7, 29.6, 29.6, 29.4, 29.3–29.2, 29, 28.9, 22.7, 14.2; HRMS (ESI) calcd for C<sub>32</sub>H<sub>67</sub>NO<sub>2</sub>S<sub>2</sub> [M + H]<sup>+</sup>: 562.4686, found: 562.4664.

#### 2.4.1.3.24. Characterization of Lipid 2.15b

Yield 83%. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 3.92–3.88 (m, 1H), 3.75–3.66 (m, 2H), 3.56–3.45 (m, 2H), 3.00–2.96 (m, 1H), 2.90–2.85 (m, 1H), 2.82–2.78 (m, 1H), 2.62–2.58 (m, 2H), 2.57–2.55 (m, 2H), 2.50–2.43 (m, 1H), 2.32 (s, 6H), 2.30–2.28 (m, 1H), 1.62–1.57 (m, 4H), 1.39–1.37 (m, 4H), 1.27 (s, 54H), 0.89 (t, *J* = 6.9 Hz, 6H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 73.7, 73.6, 73.2, 66.7, 61.8, 45.6, 34.8, 33.2, 31.9, 31.7, 29.9,

29.7, 29.7, 29.7, 29.6, 29.6, 29.5, 29.3, 29.2, 28.9, 22.7, 14.1; HRMS (ESI) calcd for  $C_{40}H_{83}NO_2S_2$   $[M + H]^+$ : 674.5938, found: 674.5935.

#### 2.4.1.3.25. Characterization of Lipid 2.16a

Yield 84%.  $^1H$  NMR (600 MHz,  $CDCl_3$ )  $\delta_{ppm}$  4.61–4.56 (m, 1H), 3.89–3.85 (m, 1H), 3.79–3.75 (m, 1H), 3.68–3.60 (m, 1H), 3.58–3.56 (m, 1H), 3.55–3.52 (m, 1H), 3.48–3.45 (m, 9H), 2.95–2.89 (m, 1H), 2.82–2.74 (m, 2H), 2.60–2.50 (m, 4H), 1.59–1.54 (m, 4H), 1.37–1.34 (m, 4H), 1.30–1.22 (m, 36H), 0.87 (t, 6H);  $^{13}C$  NMR (151 MHz,  $CDCl_3$ )  $\delta_{ppm}$  72.7, 72.0, 69.2, 64.6, 64.5, 55.4, 45.5, 35.1, 33.3, 31.9, 29.9, 29.7, 29.7, 29.7, 29.6, 29.6, 29.4, 29.3, 29.0, 28.9, 22.7, 14.17; HRMS (ESI) calcd for  $C_{33}H_{70}NO_2S_2$   $[M + H]^+$ : 577.4921, found: 577.4921.

#### 2.4.1.3.26. Characterization of Lipid 2.16b

Yield 85%.  $^1H$  NMR (600 MHz,  $CDCl_3$ )  $\delta_{ppm}$  4.60–4.53 (m, 1H), 3.87–3.85 (m, 1H), 3.79–3.74 (m, 1H), 3.67–3.59 (m, 1H), 3.58–3.54 (m, 1H), 3.54–3.51 (m, 1H), 3.47 (s, 9H), 2.94–2.89 (m, 1H), 2.81–2.74 (m, 2H), 2.58–2.51 (m, 4H), 1.59–1.53 (m, 4H), 1.37–1.33 (m, 4H), 1.26–1.22 (m, 36H), 0.87 (t, 6H);  $^{13}C$  NMR (151 MHz,  $CDCl_3$ )  $\delta_{ppm}$  72.6, 72.1, 69.2, 65.9, 64.5, 55.4, 45.5, 35.0, 33.3, 31.9, 29.9, 29.7, 29.7, 29.7, 29.7, 29.6, 29.4, 29.3, 29.0, 28.9, 22.7, 14.16; HRMS (ESI) calcd for  $C_{41}H_{86}NO_2S_2$   $[M]^+$ : 688.6100, found: 688.6091.

#### 2.4.1.3.27. Characterization of Lipid 2.17a

Yield 70%.  $^1H$  NMR (600 MHz,  $CDCl_3$ )  $\delta_{ppm}$  4.65–4.50 (m, 1H), 4.23–4.10 (m, 6H), 3.81–3.62 (m, 2H), 3.56–3.46 (m, 2H), 3.45–3.41 (m, 1H), 3.21–3.16 (m, 1H), 2.97–2.92 (m, 1H), 2.80–2.76 (m, 2H), 2.58–2.52 (m, 4H), 1.59–1.54 (m, 4H), 1.47 (t, 1H), 1.38–1.35 (m, 4H), 1.28–1.25 (m, 35H), 0.87 (t, 3H);  $^{13}C$  NMR (151 MHz,  $CDCl_3$ )  $\delta_{ppm}$  75.7, 73.0, 72.4, 69.5, 67.7, 64.7, 59.1, 46.4, 45.7, 39.5, 35.2, 33.5, 32.2, 30.0, 29.9, 29.9, 29.8, 29.7, 29.6, 29.0, 29.2, 23.0, 14.4; HRMS (ESI) calcd for  $C_{34}H_{73}N_2O_2S_2$   $[M]^+$ : 605.5113, found: 605.5110.

#### 2.4.1.3.28. Characterization of Lipid 2.17b

Yield 82%.  $^1H$  NMR (600 MHz,  $CDCl_3$ )  $\delta_{ppm}$  4.17–4.68–4.55 (m, 1H), 4.18–4.10 (m, 6H), 3.80–3.57 (m, 2H), 3.48–3.42 (m, 3H), 2.96–2.80 (m, 1H), 2.79–2.74 (m, 2H),

2.63–2.45 (m, 4H), 2.04 (s, 4H), 1.69–1.54 (m, 4H), 1.40–1.33 (m, 4H), 1.30–1.22 (m, 62H), 0.87 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  75.7, 73.0, 72.4, 69.5, 67.7, 64.7, 59.1, 46.4, 45.7, 39.5, 35.2, 33.5, 32.2, 30.0, 29.9, 29.9, 29.8, 29.7, 29.6, 29.0, 29.2, 23.0, 14.4; HRMS (ESI) calcd for  $\text{C}_{42}\text{H}_{89}\text{N}_2\text{O}_2\text{S}_2$   $[\text{M} + \text{H}]^+$ : 717.6365, found: 717.6340.

#### 2.4.1.3.29. Characterization of Lipid 2.18a

Yield 81%.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  4.78 (s, 1H), 4.41 (s, 1H), 4.30–4.28 (m, 2H), 4.13–4.06 (m, 1H), 3.77–3.59 (m, 3H), 3.40 (s), 3.47–3.29 (m, 6H), 2.93–2.88 (m, 1H), 2.82–2.74 (m, 2H), 2.59–2.52 (m, 4H), 1.92–1.85 (m, 1H), 1.59–1.54 (m, 4H), 1.37–1.34 (m, 4H), 1.31–1.20 (m, 38H), 0.87 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  169.4, 72.7, 72.6, 66.8, 64.0, 54.1, 53.1, 45.6, 34.8, 33.1, 31.9, 31.7, 30.0, 29.8, 29.7, 29.7, 29.6, 29.4, 29.1, 29.0, 22.7, 14.1; HRMS (ESI) calcd for  $\text{C}_{34}\text{H}_{70}\text{NO}_4\text{S}_2$   $[\text{M}]^+$ : 620.4746, found: 620.4736.

#### 2.4.1.3.30. Characterization of Lipid 2.18b

Yield 83%.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  4.38–4.32 (m, 1H), 4.17–4.07 (m, 1H), 3.82–3.70 (m, 1H), 3.68–3.50 (m, 3H), 3.40–3.32 (m, 6H), 2.94–2.89 (m, 1H), 2.83–2.76 (m, 2H), 2.62–2.53 (m, 4H), 1.61–1.56 (m, 4H), 1.39–1.36 (m, 4H), 1.32–1.22 (m, 51H), 0.91–0.88 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  169.3, 72.6, 72.5, 66.7, 64.0, 54.1, 53.0, 45.0, 34.8, 33.0, 31.8, 31.7, 29.9, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.0, 22.6, 14.0; HRMS (ESI) calcd for  $\text{C}_{42}\text{H}_{86}\text{NO}_4\text{S}_2$   $[\text{M}]^+$ : 732.5998, found: 732.5988.

#### 2.4.1.3.31. Characterization of Lipid 2.19a

Yield 81%.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  4.54–4.53 (m, 1H), 4.20–4.15 (m, 4H), 4.05–3.86 (m, 4H), 3.80–3.64 (m, 4H), 3.59–3.52 (m, 2H), 3.43 (s, 3H), 2.97–2.92 (m, 1H), 2.83–2.75 (m, 2H), 2.60–2.53 (m, 4H), 2.04 (s, 1H), 1.63–1.53 (m, 4H), 1.36–1.35 (m, 1H), 1.29–1.23 (m, 38H), 0.87 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  72.6, 72.5, 72.4, 66.0, 65.9, 65.3, 65.2, 56.0, 51.8, 45.4, 34.9, 33.2, 31.9, 31.7, 31.6, 29.9, 29.7, 29.7, 29.6, 29.4, 29.3, 29.0, 28.9, 22.7, 14.1; HRMS (ESI) calcd for  $\text{C}_{35}\text{H}_{74}\text{NO}_4\text{S}_2$   $[\text{M}]^+$ : 636.5059, found: 636.5055.

#### 2.4.1.3.32. Characterization of Lipid 2.19b



Yield 85%.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  4.55–4.50 (m, 1H), 4.21–4.14 (m, 3H), 3.97–3.86 (m, 4H), 3.78–3.71 (m, 3H), 3.68–3.63 (m, 1H), 3.61–3.50 (m, 1H), 3.43 (s, 3H), 2.96–2.91 (m, 1H), 2.82–2.75 (m, 2H), 2.67–2.51 (m, 4H), 1.87–1.84 (m, 1H), 1.62–1.53 (m, 4H), 1.37–1.34 (m, 4H), 1.30–1.22 (m, 51H), 0.87 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  72.6, 72.5, 72.4, 68.0, 66.1, 65.9, 65.3, 64.5, 56.0, 53.4, 51.8, 46.2, 45.4, 34.9, 33.2, 31.9, 31.7, 31.6, 29.9, 29.7, 29.7, 29.6, 29.4, 29.3, 29.0, 28.9, 25.6, 22.7, 14.1; HRMS (ESI) calcd for  $\text{C}_{43}\text{H}_{90}\text{NO}_4\text{S}_2$   $[\text{M} + \text{H}]^+$ : 749.6384, found: 749.6382.

#### 2.4.1.3.33. Characterization of Control Lipid 2.20a

Yield 89%.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  3.96–3.93 (m, 1H), 3.55 (s, 9H), 3.53–3.49 (m, 1H), 3.31–3.27 (m, 1H), 3.20–3.16 (m, 1H), 3.06–3.03 (m, 1H), 2.96 (s, 1H), 2.88 (s, 1H), 2.77–2.64 (m, 4H), 1.65–1.57 (m, 4H), 1.42–1.63 (m, 4H), 1.30–1.22 (m, 40H), 0.87 (t, 6H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  70.0, 55.7, 46.4, 39.7, 37.4, 33.5, 32.1, 31.7, 29.9, 29.8, 29.7, 29.7, 29.6, 29.5, 29.5, 29.4, 29.2, 29.0, 22.9, 14.3; HRMS (ESI) calcd for  $\text{C}_{30}\text{H}_{64}\text{NS}_2$   $[\text{M}]^+$ : 502.9685, found: 502.9676.

#### 2.4.2. Preparation of Liposomes

The liposomes were prepared by the thin-film hydration method in Milli-Q water. The synthesized lipids [stock solution of chloroform/methanol at 8:2 (v/v)] were mixed with PE [stock solution of chloroform/methanol at 8:2 (v/v)] with a mole ratio of 1:1 in a glass vial and dried under reduced pressure for 6 h to formulate the lipid thin film. The PE lipid was used as the helper lipid. After that, the thin film was hydrated with Milli-Q water overnight at 55 °C (final concentration of the liposome was 1 mM), and the solution was vortexed for 15 min. Finally, the solution was sonicated for 10 times (30 s of sonication followed by 30 s of cooling on ice) until the thin film disappeared.<sup>26</sup>

#### 2.4.3. Transmission Electron Microscopy imaging

For transmission electron microscopy (TEM) imaging, vesicles were prepared by the method described above in 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 100 mM KCl.<sup>26</sup> The prepared vesicles (no extrusion method was used) were diluted to half of its original concentration using HEPES buffer, pH 7.4. Onto a carbon-coated copper grid, a 10  $\mu\text{L}$  solution of liposome

was placed from the diluted solution and allowed to absorb for 1 min. After that, the grid was carefully blotted with filter paper, keeping only a trace amount of the solution in the middle of the grid. Then, the grid was allowed to dry for 10 min at 30 °C. Finally, 5–10  $\mu$ L of 1% uranyl acetate solution (in water) was added to the grid and allowed to dry for another 1 min. The excess uranyl acetate solution was wicked off, and the grid was dried overnight at 30 °C. The images of the vesicles formed on the carbon-coated copper grid were collected using a JEOL JEM 2100 transmission electron microscope (operated at a maximum accelerating voltage of 200 kV).

#### 2.4.4. Particle Size and Surface Change of the Lipoplexes

The particle size and surface potential of free liposomes and lipoplex (at various N/P ratios) were characterized by using dynamic light scattering (DLS) and  $\zeta$  potential measurements (Zetasizer Nano ZS90, Malvern, Westborough, MA), respectively, at 25 °C.

#### 2.4.5. Isoelectric Point Measurements

For the estimation of the isoelectric point (IEP) of the synthesized lipids, the surface potential of the lipid was measured in various pH solutions. The liposomes were prepared according to the previously discussed thin-film hydration method, using 5 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.6, containing 5 mM NaCl. For the  $\zeta$  measurements, isosmotic buffers consisting of 10 mM buffering agent and 10 mM salt at different pH values ranging from 3.0 to 9.0 were freshly prepared. The buffers with a pH range of 3.0–6.5 were prepared using citric acid and trisodium citrate. 3-(*N*-morpholino)propanesulfonic acid (MOPS) was used for buffer with pH 7.0, and tris(hydroxymethyl)aminomethane (Tris)–HCl was used to prepare buffer with pH values in the range of 7.5–9.0. Disposable capillary cells (DTS1061) and fluorescence cuvettes were used for  $\zeta$  potential and DLS measurements. All of the analyses were performed at least three times per sample, and the corresponding polydispersity indexes (PDIs) were within 0.1–0.4.

#### 2.4.6. Lipid Phase-Transition Temperature Measurements

To establish the phase-transition temperature ( $T_m$ ) from the ordered gel to liquid disordered phase of the lipid bilayers, temperature-dependent steady-state

anisotropy measurements were performed.<sup>26</sup> For this purpose, environment-susceptible fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was used. Vesicles were prepared using a similar method described in the previous section (the hydration was performed by using 20 mM HEPES buffer, pH 7.4, containing 100 mM KCl). A handheld mini-extruder with a polycarbonate membrane (diameter of 100 nm) was used to prepare large unilamellar vesicles. Consequently, to the extruded vesicles (800  $\mu$ L), 8  $\mu$ L of 1 mM DPH solution in THF was added (final concentration of DPH was 10  $\mu$ M; DPH <1% v/v in vesicle solution). Under tumbling conditions at room temperature overnight, this liposomal solution was set aside for maximum incorporation of the DPH molecule inside the hydrophobic core of the lipid bilayer. The steady-state fluorescence anisotropy measurements were performed with a refrigerated system using a Peltier temperature controller connected to a Fluoromax-4 spectrofluorometer (Horiba Scientific). At the peak of the fluorescence spectrum, where  $I_{VV}$  and  $I_{VH}$  are the fluorescence intensities of the emitted light polarized parallel and perpendicular to the excited light, respectively, and  $G = I_{VH}/I_{HH}$  is the instrumental grating factor, the degree of anisotropy ( $r$ ) in the DPH fluorescence ( $\lambda_{\text{ex}} = 350$  nm;  $\lambda_{\text{em}} = 429$  nm) was calculated. All anisotropy values of the DPH probe are the mean values of three individual determinations, before and after the anisotropy measurements [ $\text{anisotropy } (r) = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH})$ ]. To explore the  $T_m$  values of the lipids, plots of the degree of anisotropy ( $r$ ) of the DPH probe as a function of temperature were studied.

#### 2.4.7. Preparation of Lipoplexes

An assorted amount of liposomes (containing synthesized lipid and PE with a mole ratio of 1:1) was mixed with a constant amount of GFP-tagged plasmid DNA (bp = 5kb) by pipetting meticulously at a variety of N/P ratios (from 0 to 8), and the mixture was incubated at 30 °C for 30 min to prepare the lipid–DNA complex (lipoplex).<sup>8, 17</sup> The theoretical N/P ratio corresponded to the charge ratio of N atoms on cationic lipid to nucleotide phosphates (in mole) and was calculated by considering the average nucleotide mass.

#### 2.4.8. Gel Retardation Assay

Gel retardation assay is widely used to investigate the formation of liposome/pDNA complex (lipoplex).<sup>8, 17</sup> The stability of lipoplexes of different N/P ratios (ranging from 0 to 8) was measured, as mentioned in the previous section. For this study, a fixed amount of pDNA was used (0.125 µg) and also a fixed amount of each lipoplex solution (20 µL) was electrophoresed on the 0.8% (w/v) agarose gel containing 1 µM ethidium bromide and Tris-acetate (TAE) running buffer at 100 V for 30 min. The pDNA was visualized with a UV lamp using a BioRad Universal Hood. The gel retardation assay in the presence of serum was also performed following similar experimental methods. The lipoplex solution (20 µL; containing 10 or 50% serum) was obtained by adding Tris-HCl buffer and serum (2 or 10 µL) and incubating for a specific time.

#### 2.4.9. Cytotoxicity Assays

The HEK-293T cells were seeded in 96-well plates to get 70% confluency after 12 h incubation at 37 °C. Different concentrations of the compound were prepared in plain Dulbecco's modified Eagle's medium (DMEM) and added to the cells directly after a phosphate-buffered saline (PBS) wash. The MTT solution was prepared at 5 mg/mL in PBS and filtered through a 0.2 µm filter. After 24 h, 100 µL of 10% diluted MTT in plain DMEM was added in each well. Cells were incubated for 4 h at 37 °C with 5% CO<sub>2</sub>, 95% air, and complete humidity. After 4 h, the MTT solution was removed and replaced with 100 µL of DMSO. The plate was further incubated for 15 min in the dark at room temperature. The optical density (OD) of the wells was determined using a Multiscan Go plate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm.

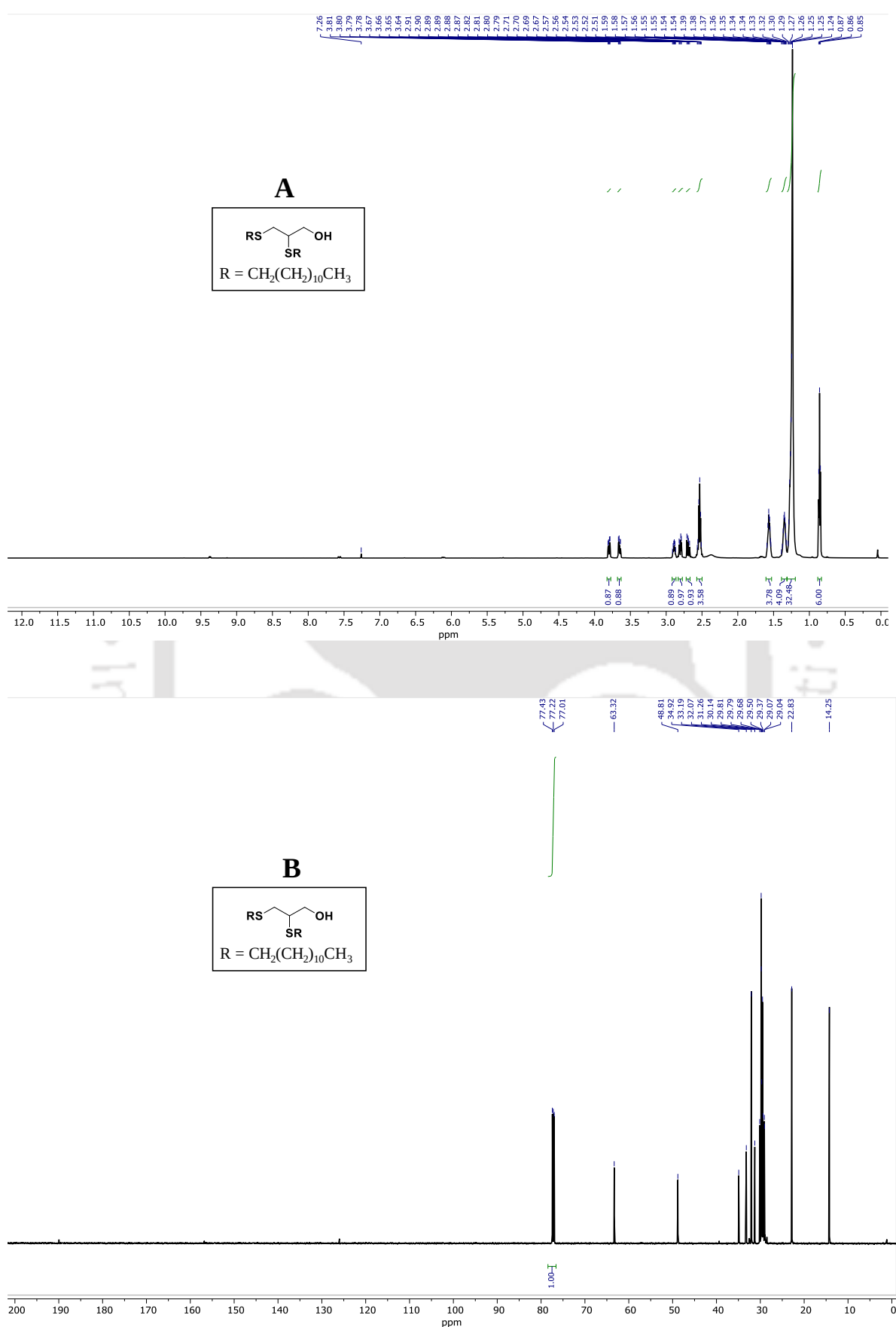
#### 2.4.10. Measurement of Transfection Efficiency

To check the transfection efficiency of the potent lipids, we used HEK-293T cells. The HEK-293T cells were seeded in 12-well plates with a density of  $0.1 \times 10^6$  per well, containing DMEM and 10% fetal bovine serum (FBS), and the plates were incubated for 12 h at 37 °C. For the transfection experiment, first, pMMP7-GFP plasmid (1.5 µg) and Opti-MEM media were added in a microfuge tube, and the final volume was adjusted to 150 µL. In another centrifuge tube, lipoplex of different N/P ratios and Opti-MEM media were added, and the final volume was adjusted to 150 µL. Then, the

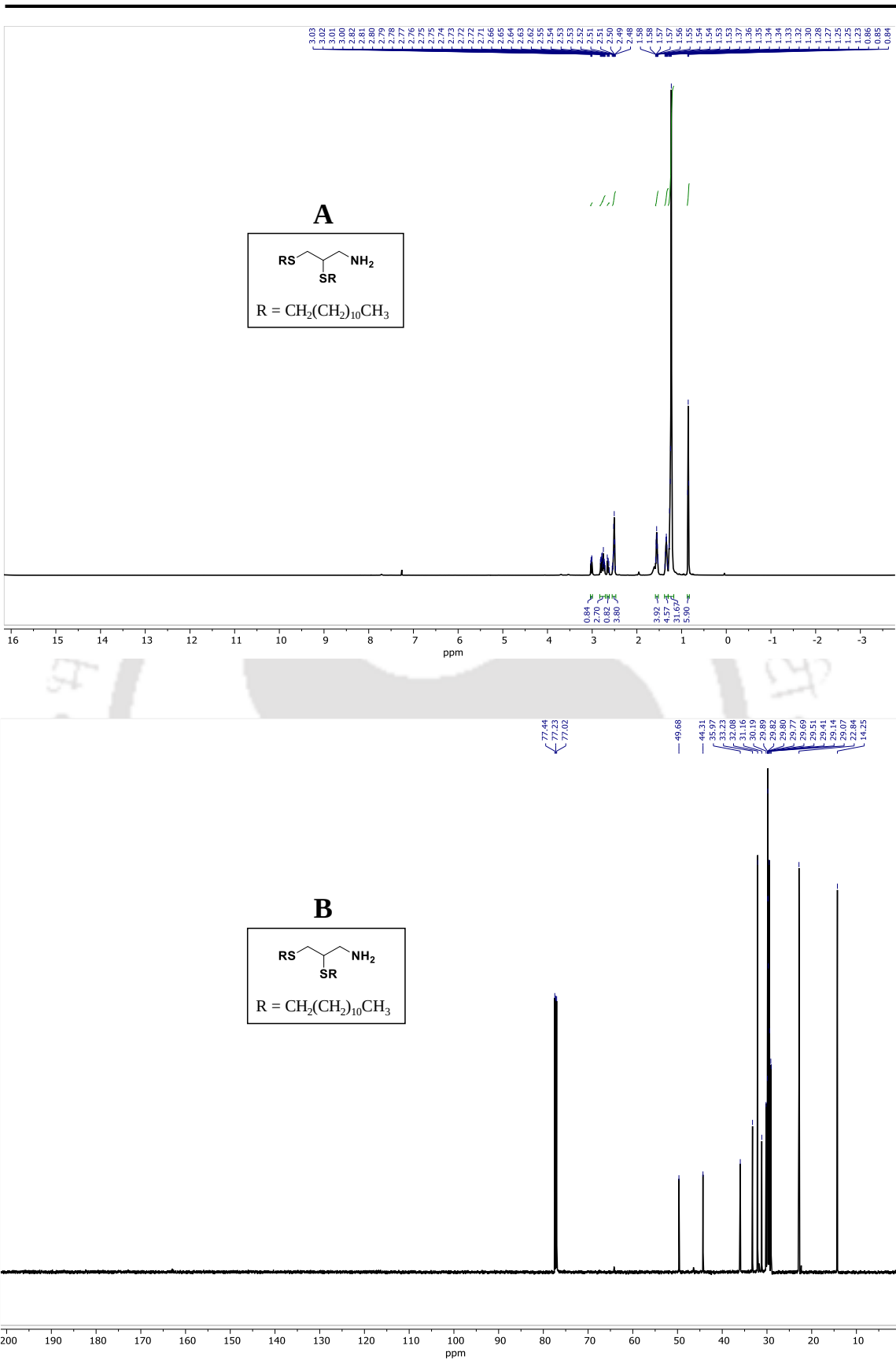
microfuge tubes were incubated for 5 min at 37 °C. After that, the solutions were mixed and kept at room temperature for 30 min. Before transfection, the media of 12-well plates was removed and the cells were washed with PBS. Then, the media containing lipoplex (300 µL) was added in each well and the cells were incubated for 4 h at 37 °C. After that, the medium was replaced with fresh DMEM with 2% FBS and incubated for another 24 h. Finally, the transfected cells were analyzed to measure GFP fluorescence-labeled cells after 24 h, under 40× magnifications with a green filter using Fluid imaging system. Lipofectamine 2000 was used as the positive control to measure the transfection efficiency. For all of the measurements, 0.2 µL of Lipofectamine 2000 from the commercially available stock was used as control (as no concentration was provided by the commercial source).

#### 2.4.11. GFP-Uptake Assays

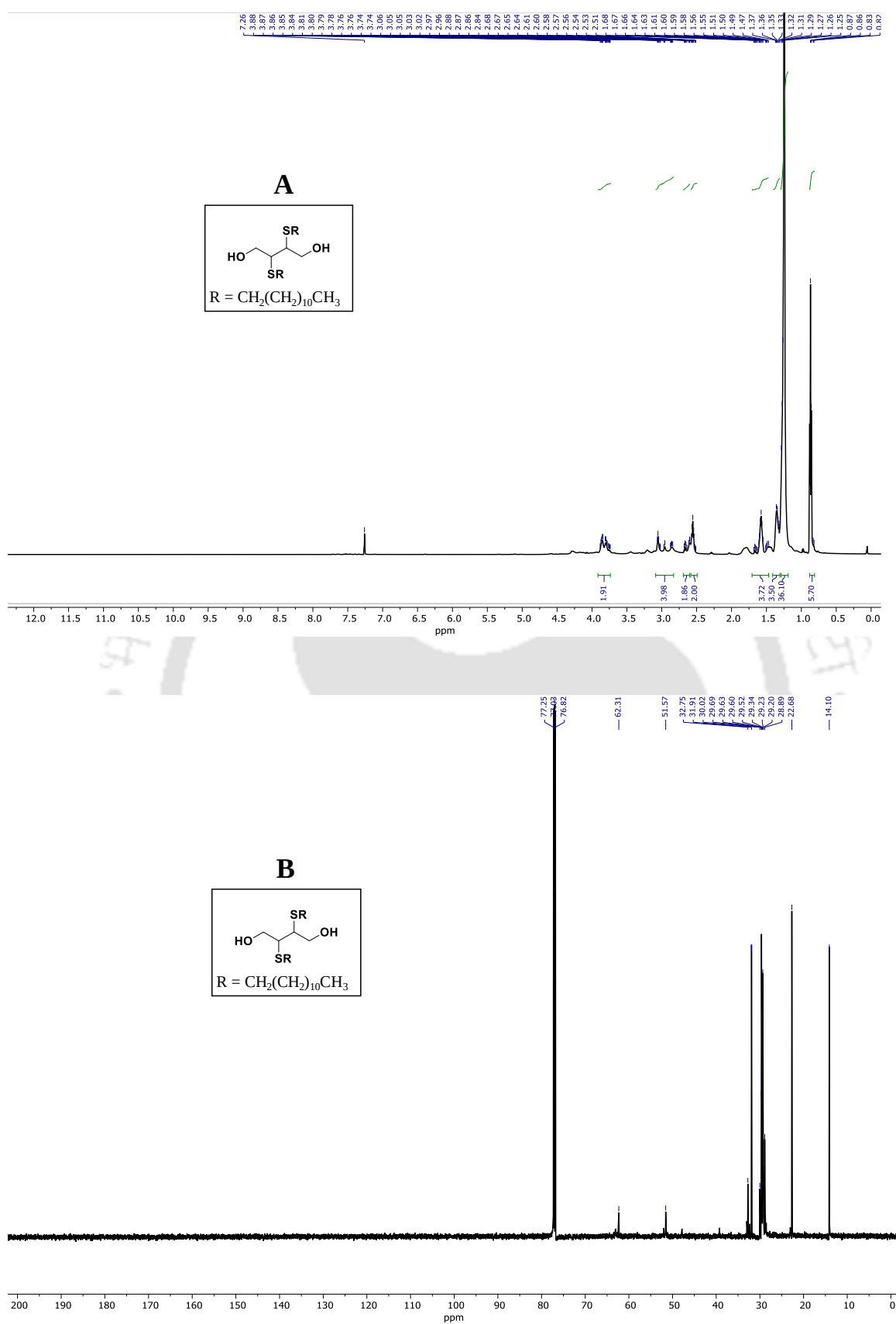
The HEK-293T cells were seeded in six-well plates (density  $0.3 \times 10^6$  per well in DMEM containing 10% FBS). The plates were then incubated overnight at 37 °C. For transfection, the pMMP7-GFP (2.0 µg) and Opti-MEM solution were added to make the final volume of 150 µL in a centrifuge tube. In a separate tube, the lipoplex (at different N/P ratios) and Opti-MEM media (to make the final volume of 150 µL) were added. Then, both the tubes were incubated for 5 min at room temperature. The solutions were mixed and kept at room temperature for 30 min. The wells of the plate were washed with PBS. Then, media containing lipoplex (300 µL) were added in each well and the cells were incubated for 4 h at 37 °C. After that, the medium was replaced with fresh DMEM with 2% FBS and incubated for another 24 h. For FACS analyses, experiments were performed on BD FACSCalibur using the FL1 channel for green fluorescence. Data analyses were done through FCS Express 6 Flow Cytometry software. Autofluorescence was detected using nontransfected cells as control, indicated as a peak in the histogram plot using FL1 channel. The M1 region is shown in plots capturing green fluorescence intensity in transfected cells, which is higher than autofluorescence intensity. Approximately 10 000 events were statistically evaluated for each sample in the histogram.

2.4.12.  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of synthesized compounds:Figure 2.10.  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 2.3a.

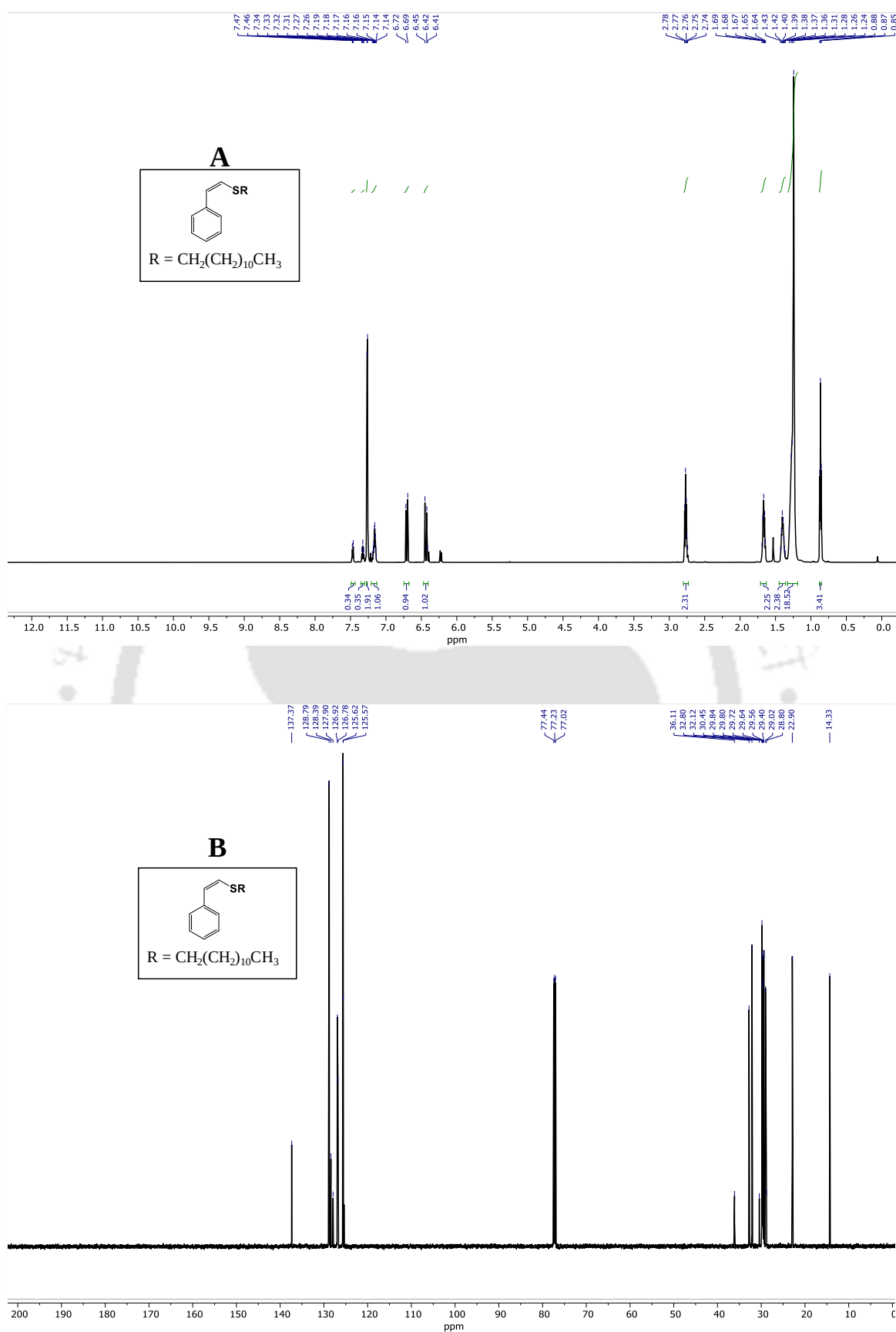




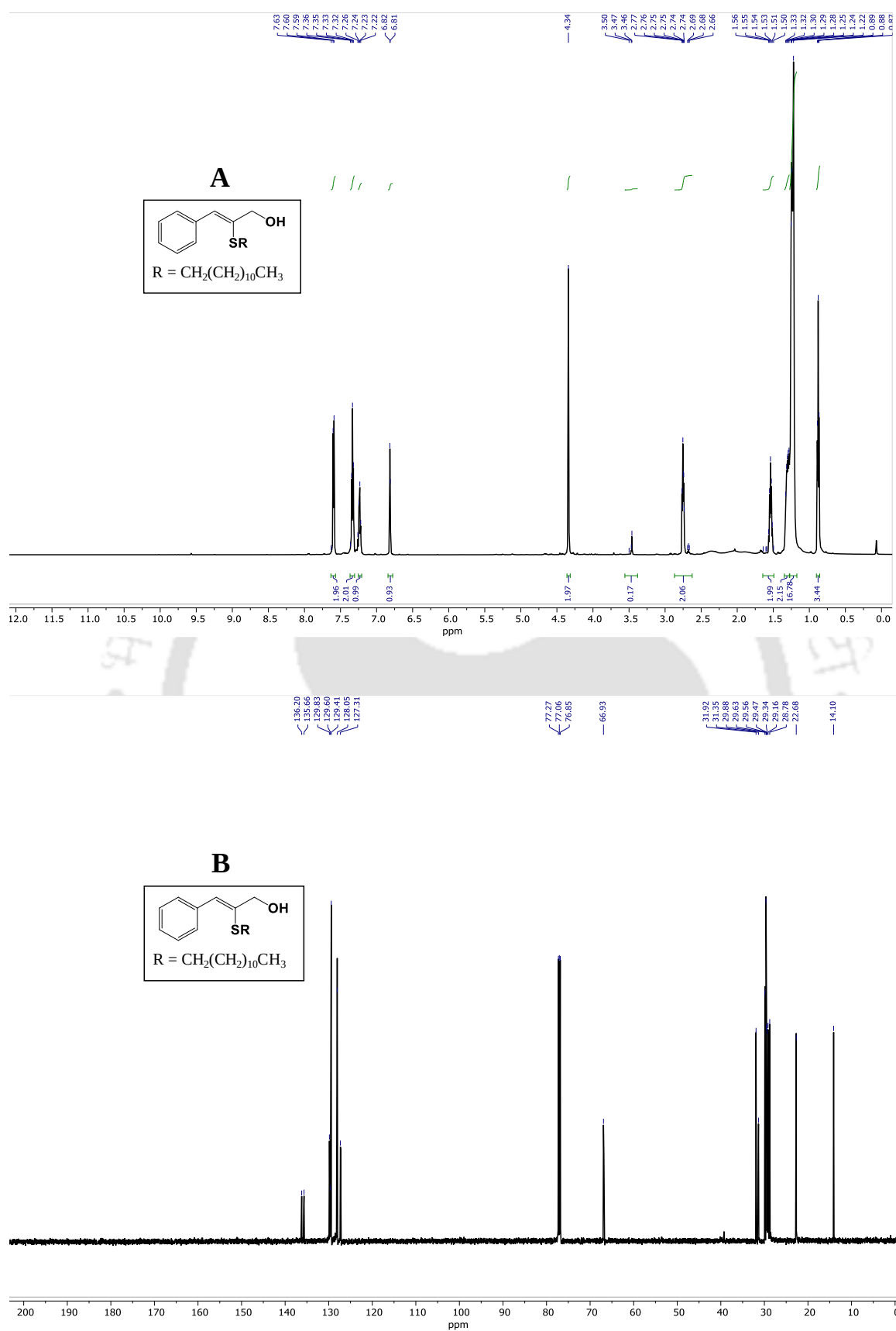
**Figure 2.11.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound **2.3b**.



**Figure 2.12.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of compound 2.3c.



**Figure 2.13.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 2.3d.



**Figure 2.14.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of compound **2.3e**.

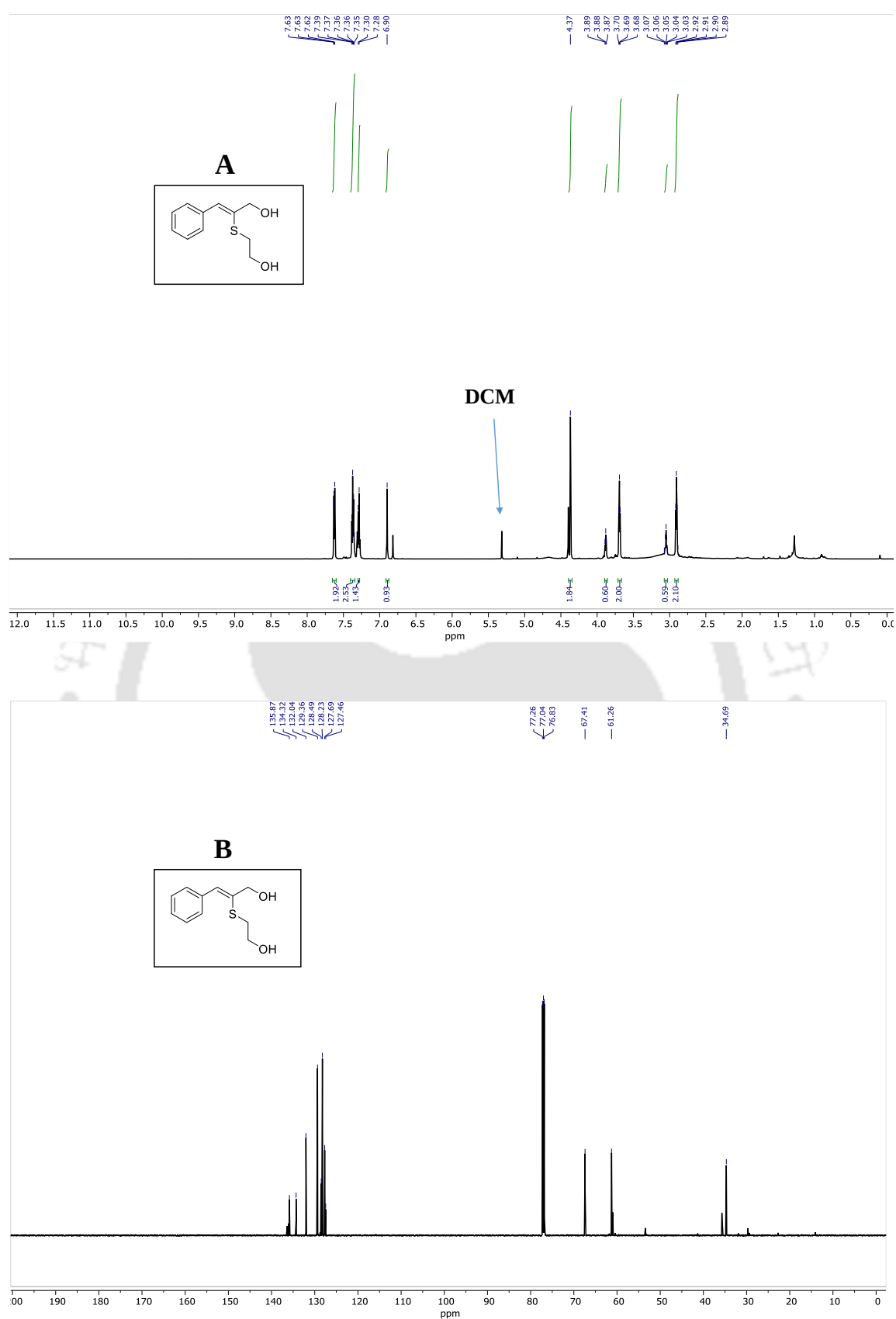
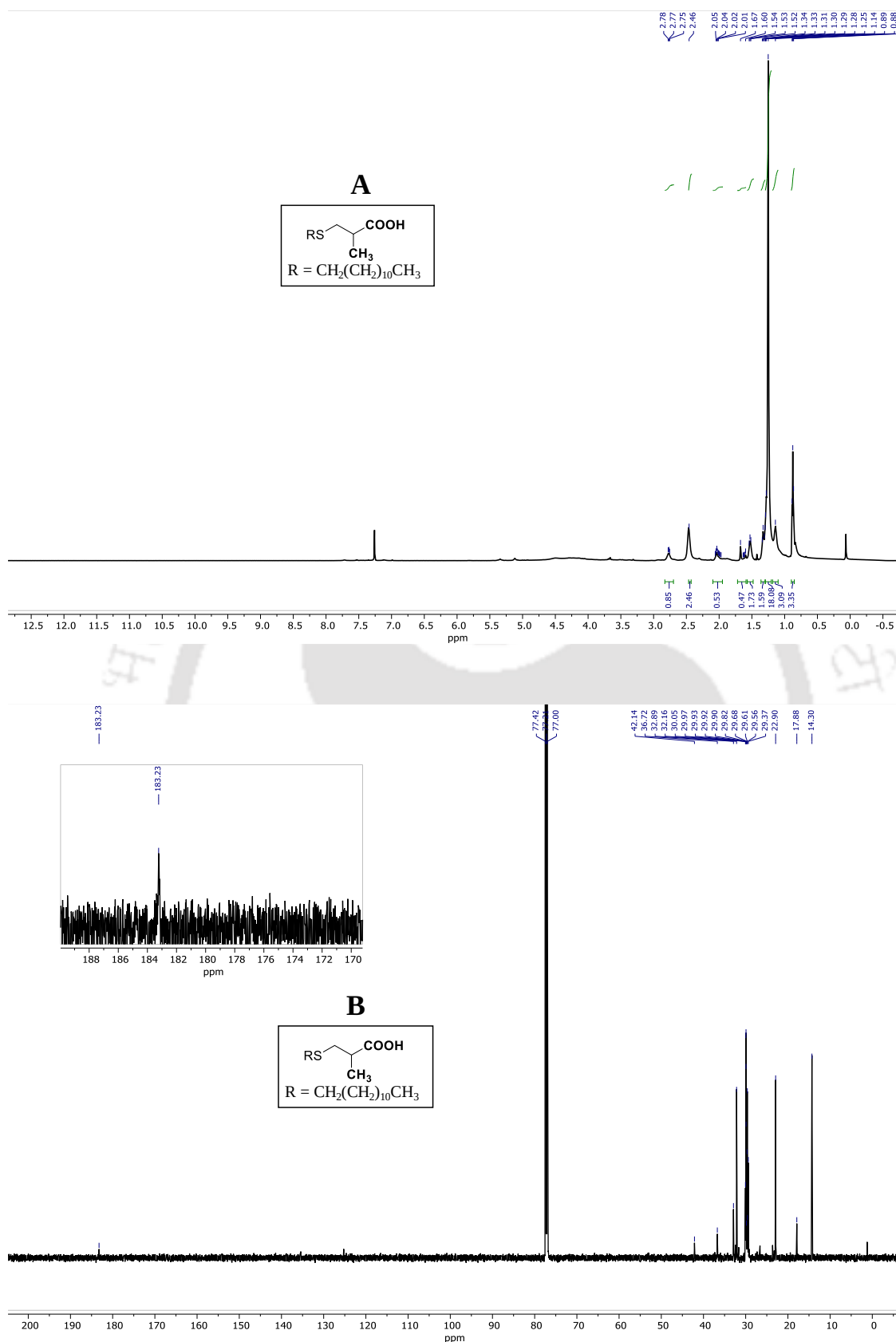
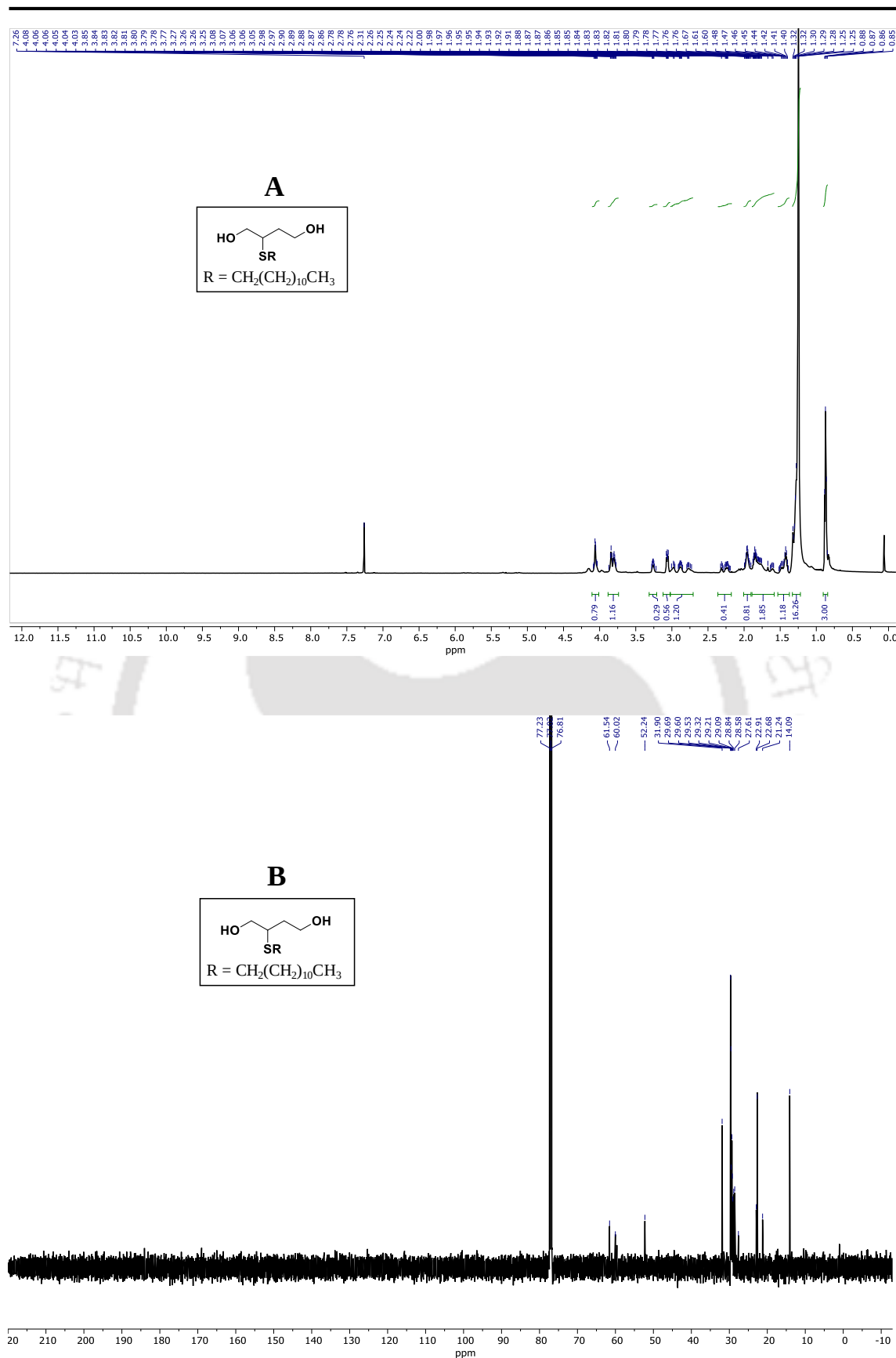


Figure 2.15. <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of compound 2.3f.

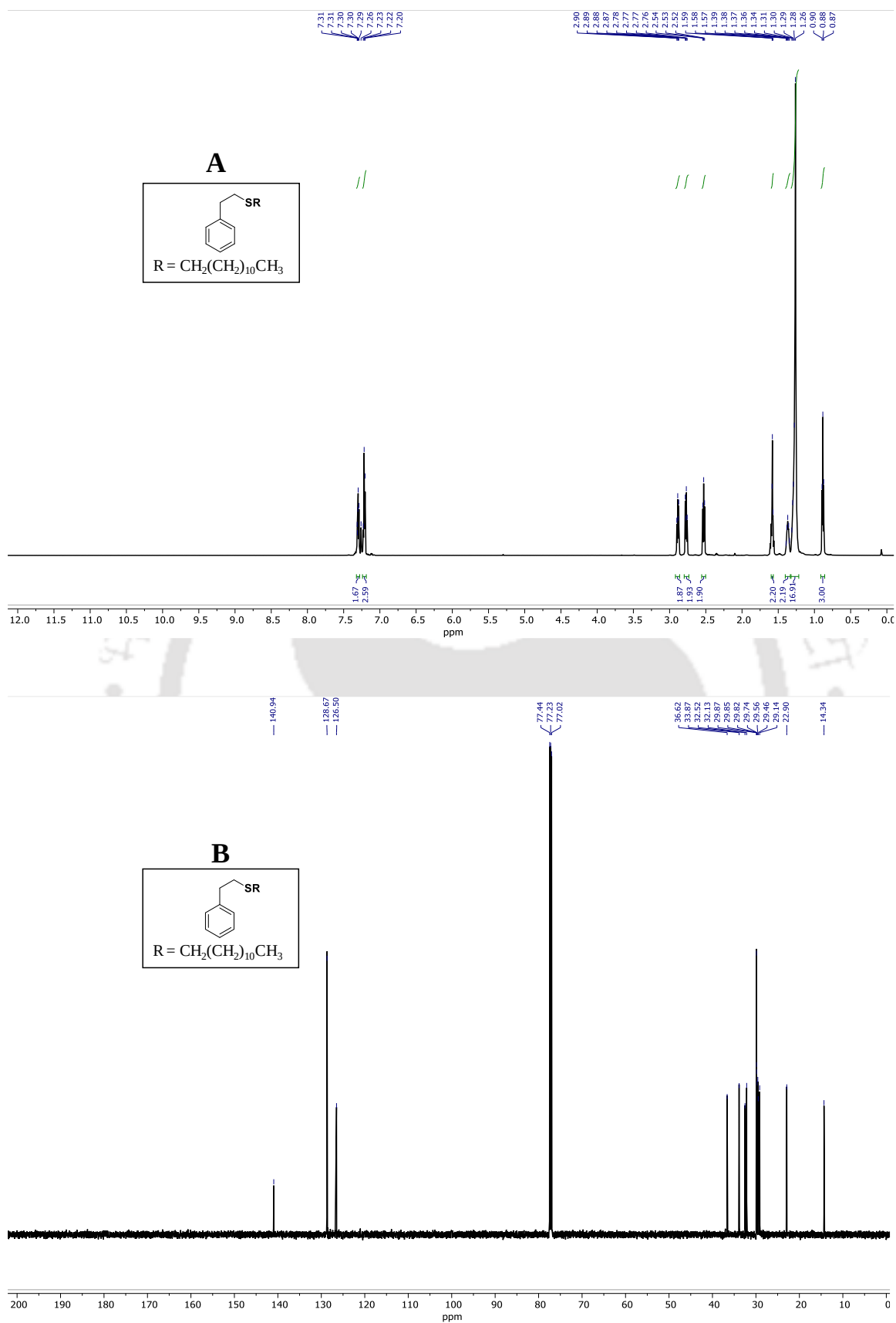


**Figure 2.16.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound **2.5a**.

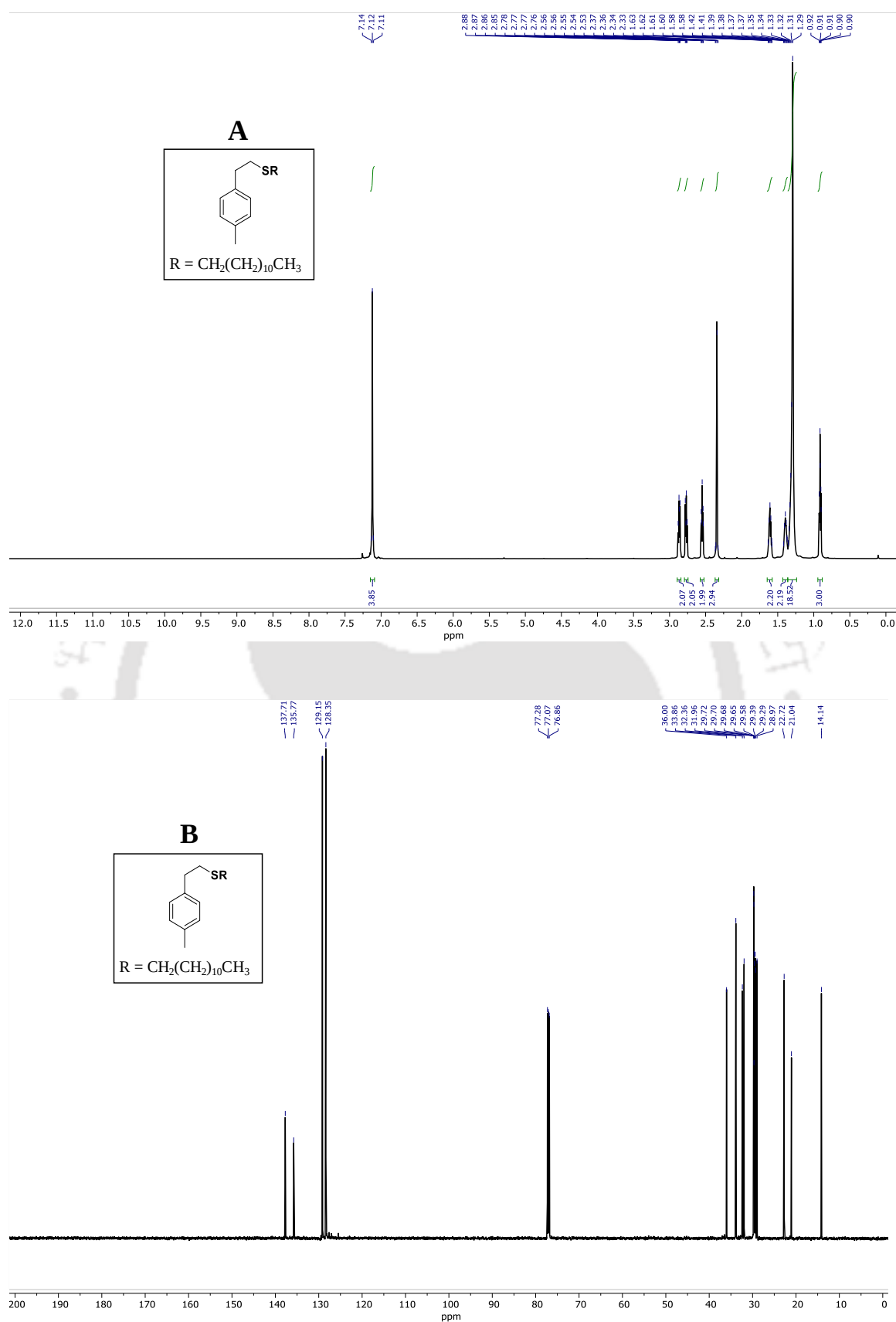


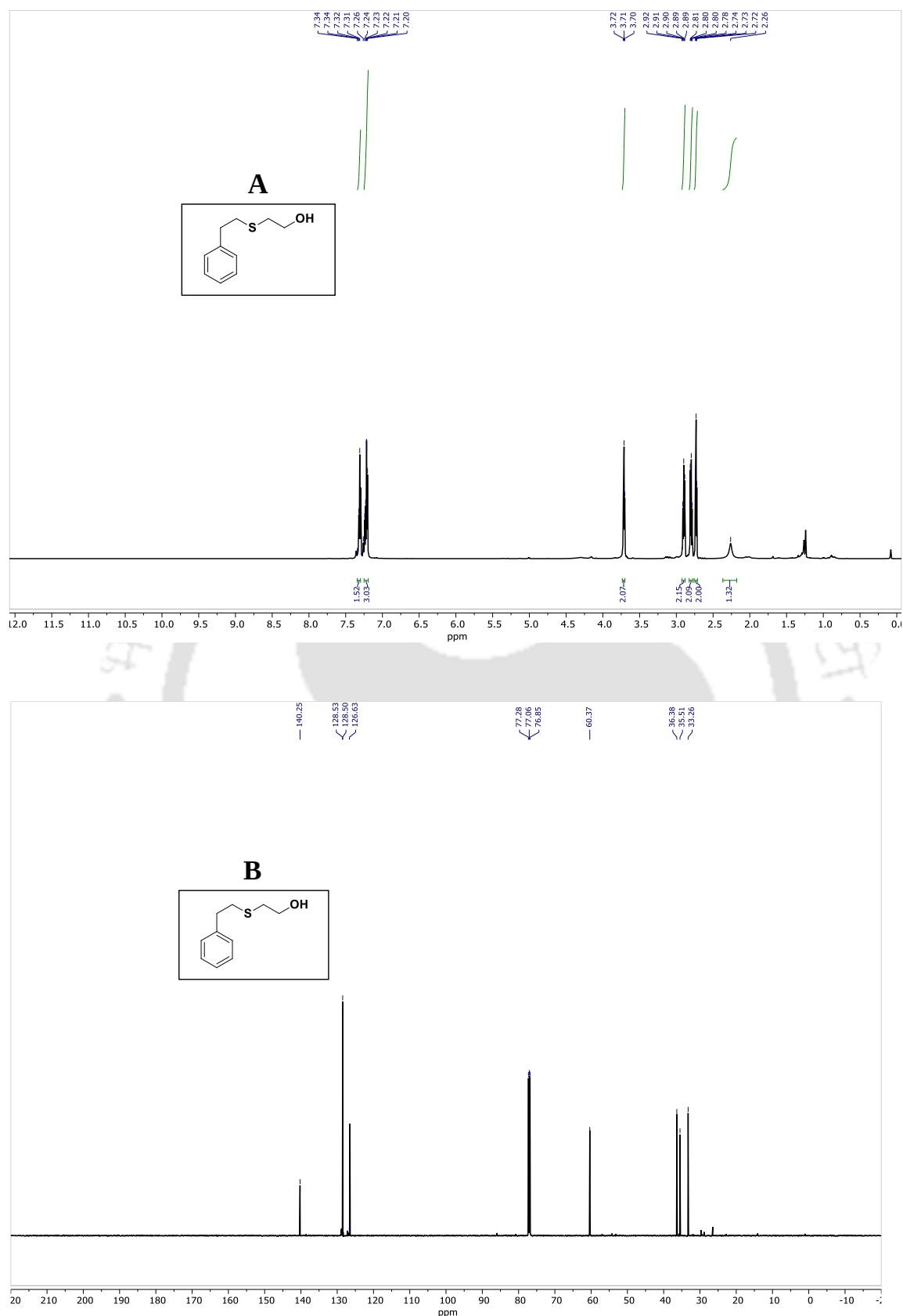


**Figure 2.17.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound **2.5b**.

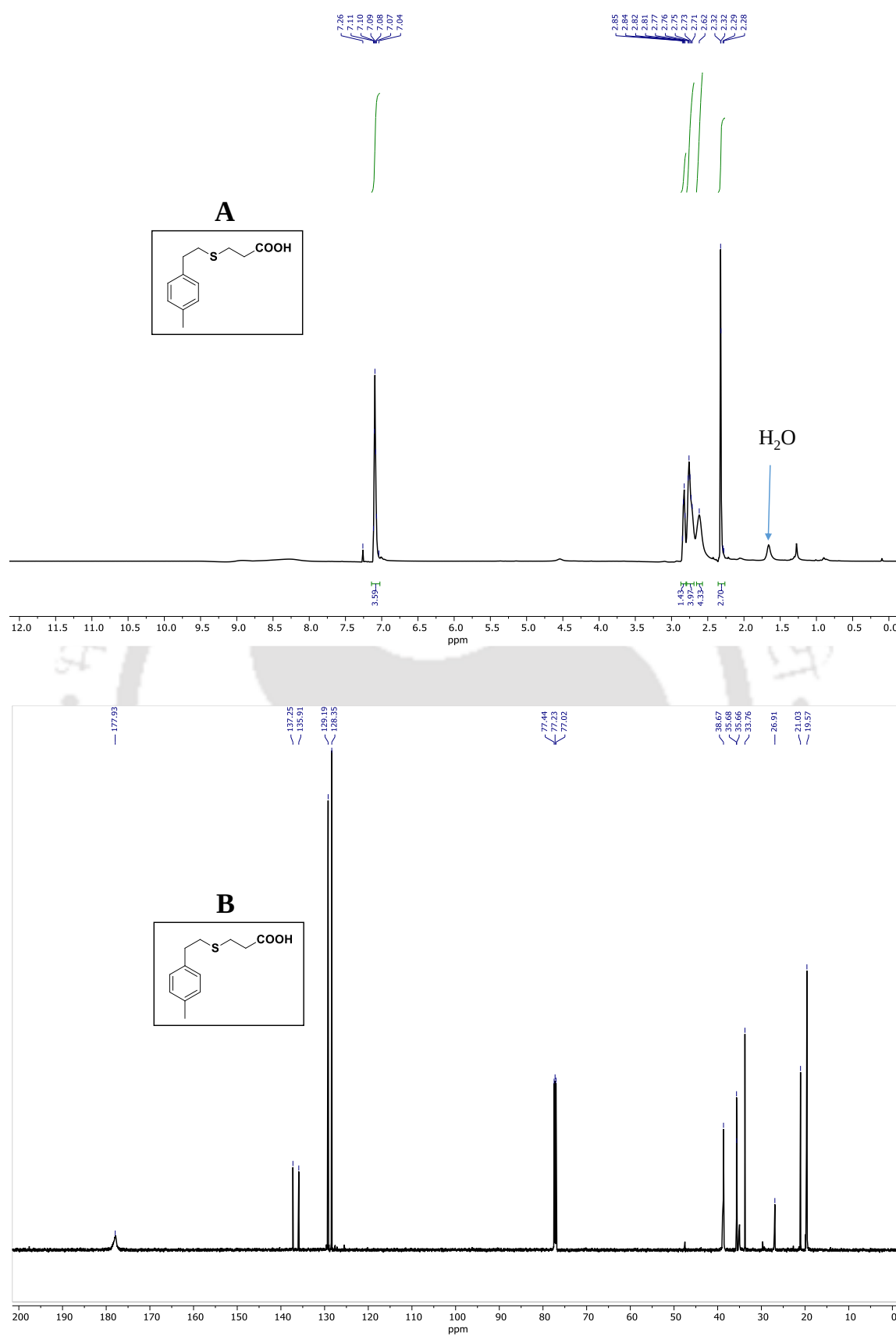


**Figure 2.18.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound **2.5c**.

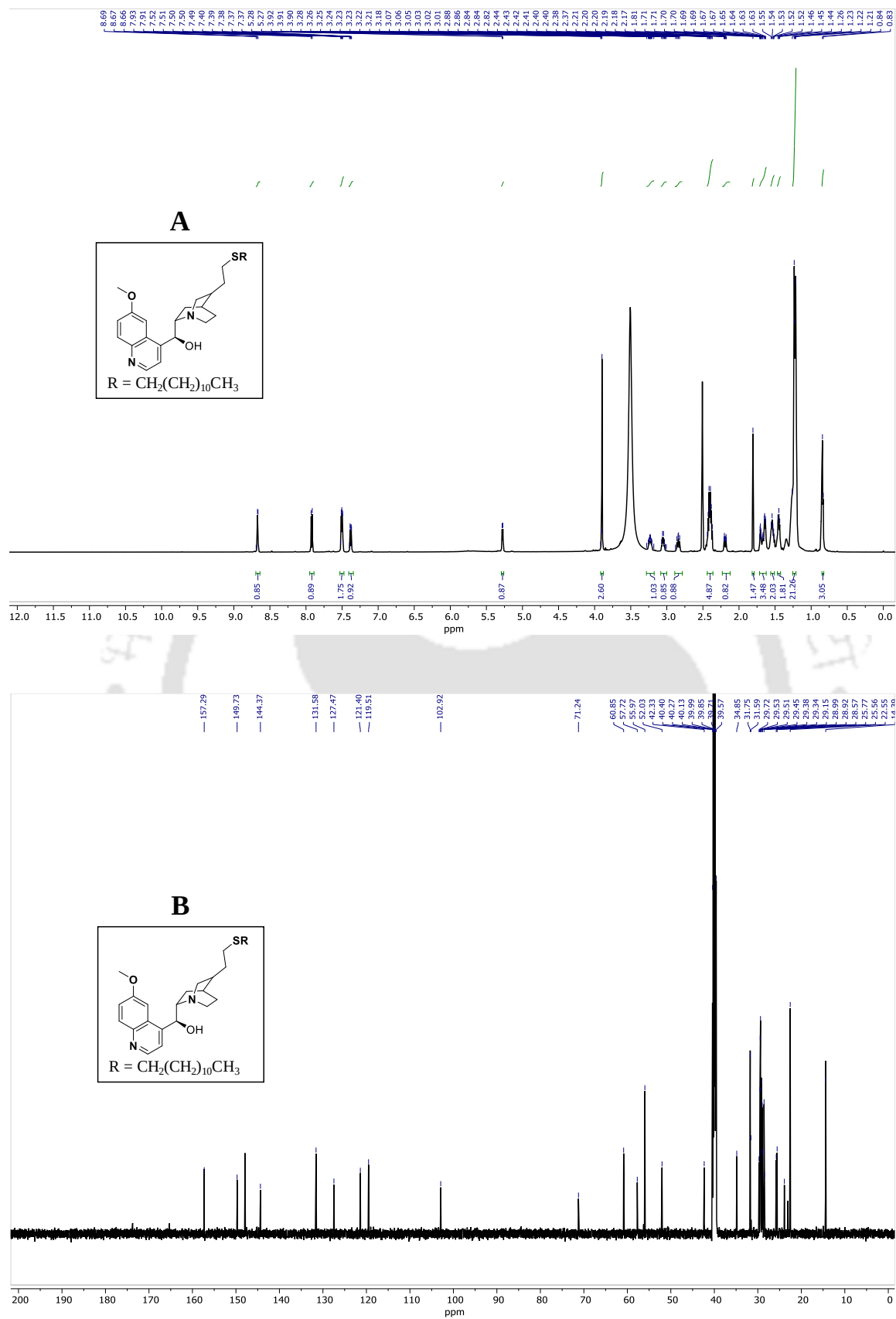




**Figure 2.20.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound **2.5e**.

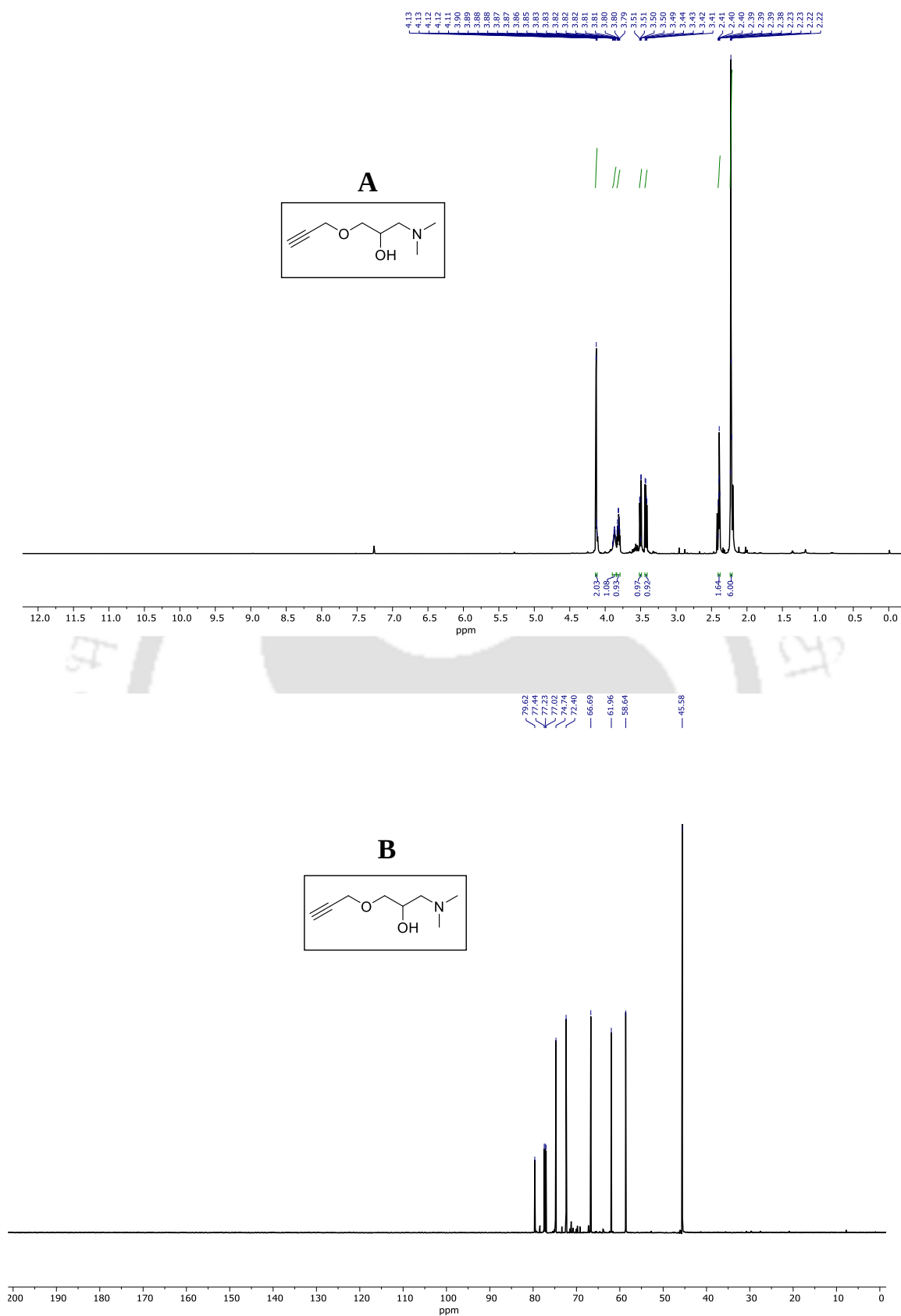


**Figure 2.21.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 2.5f.

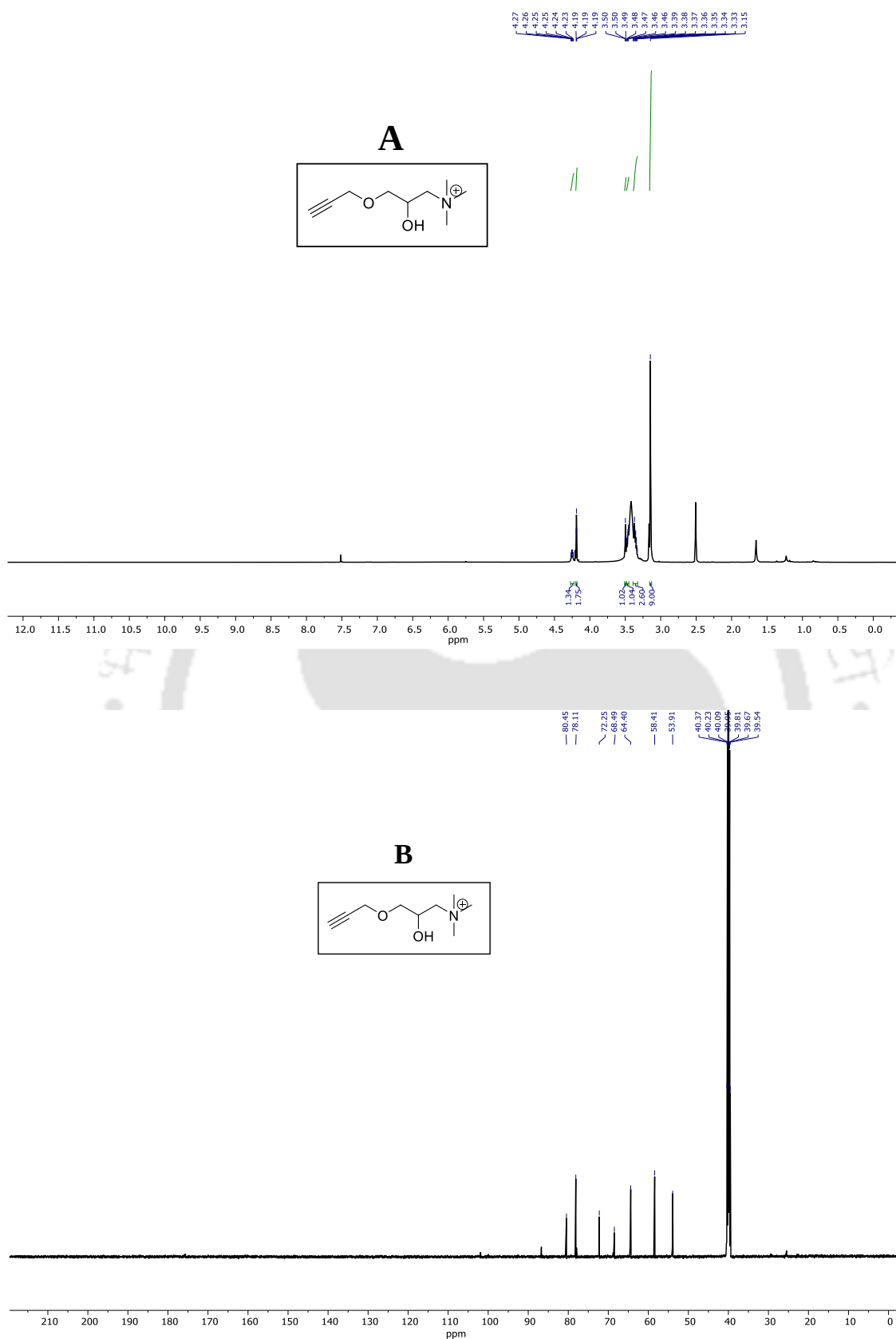


**Figure 2.22.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 2.5g.

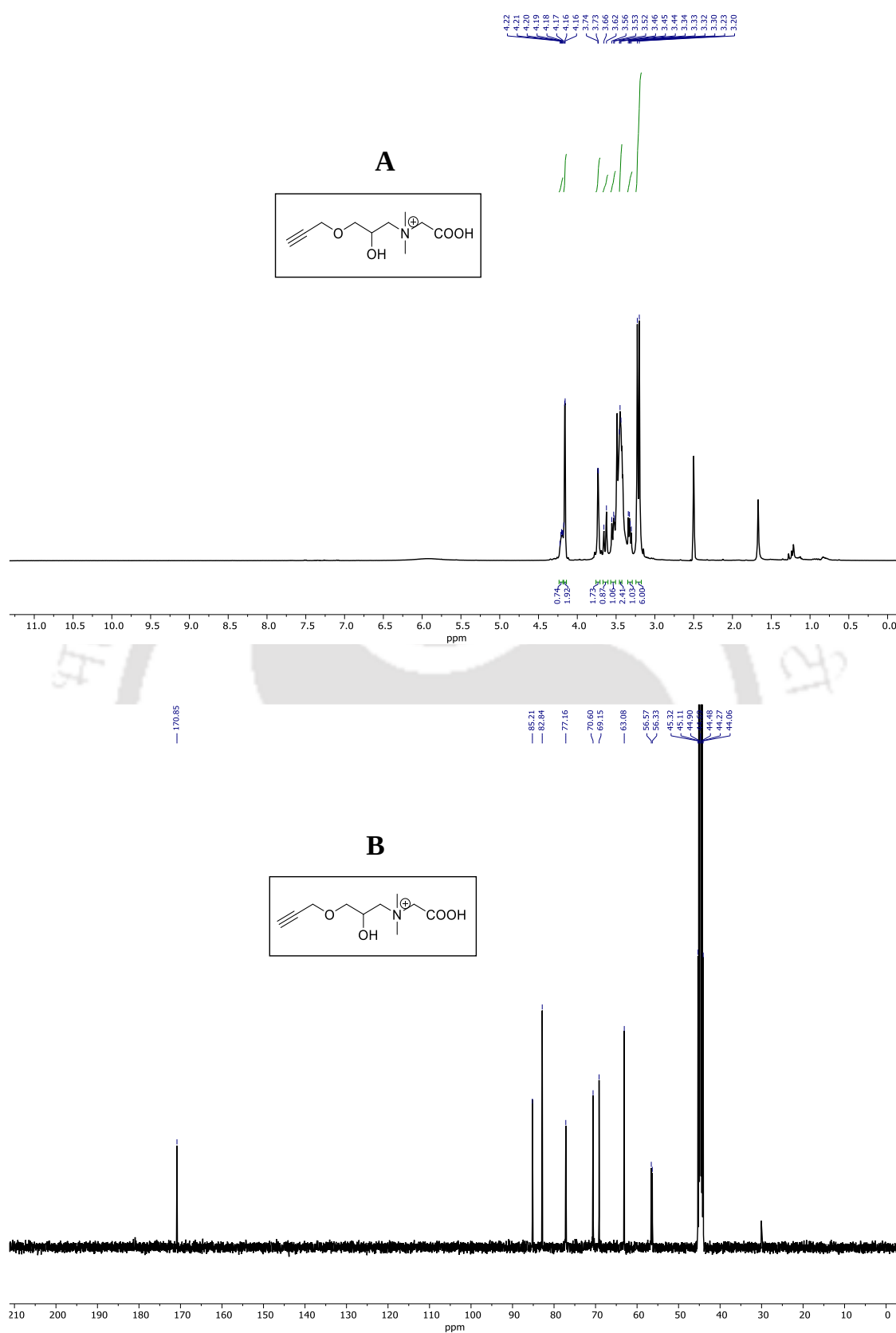




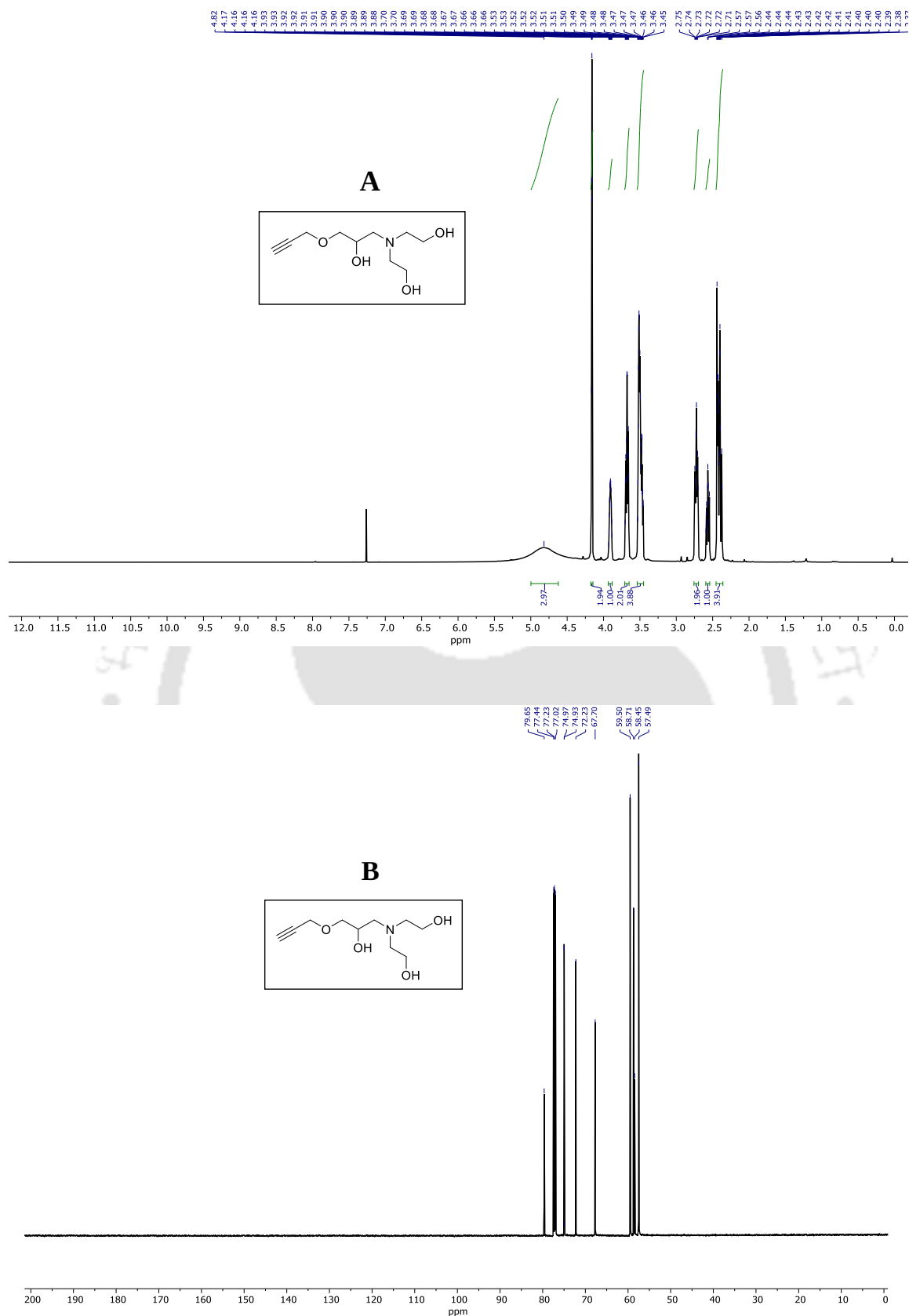
**Figure 2.23.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 2.7.



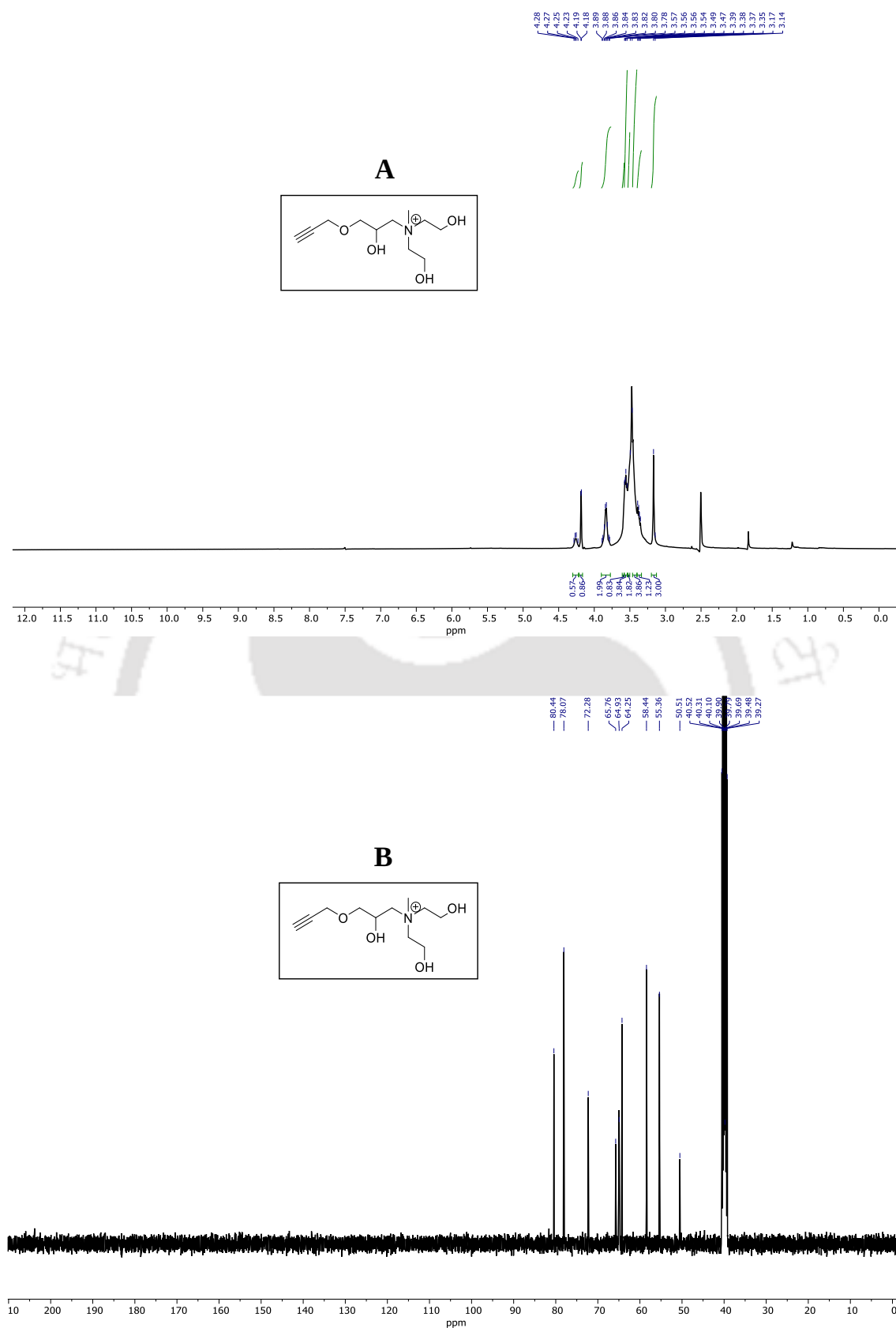
**Figure 2.24.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of compound 2.8.

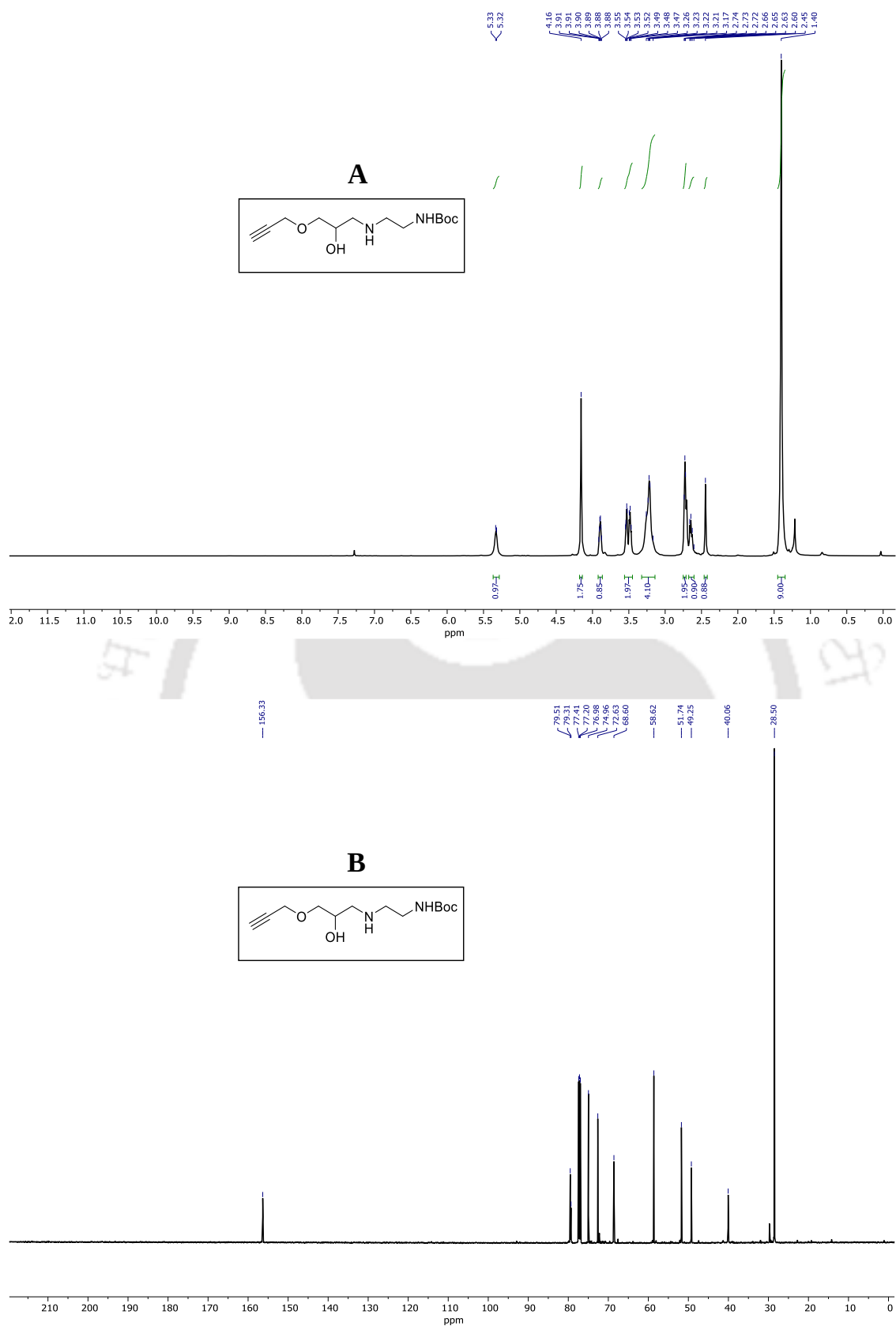


**Figure 2.25.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of compound 2.9.

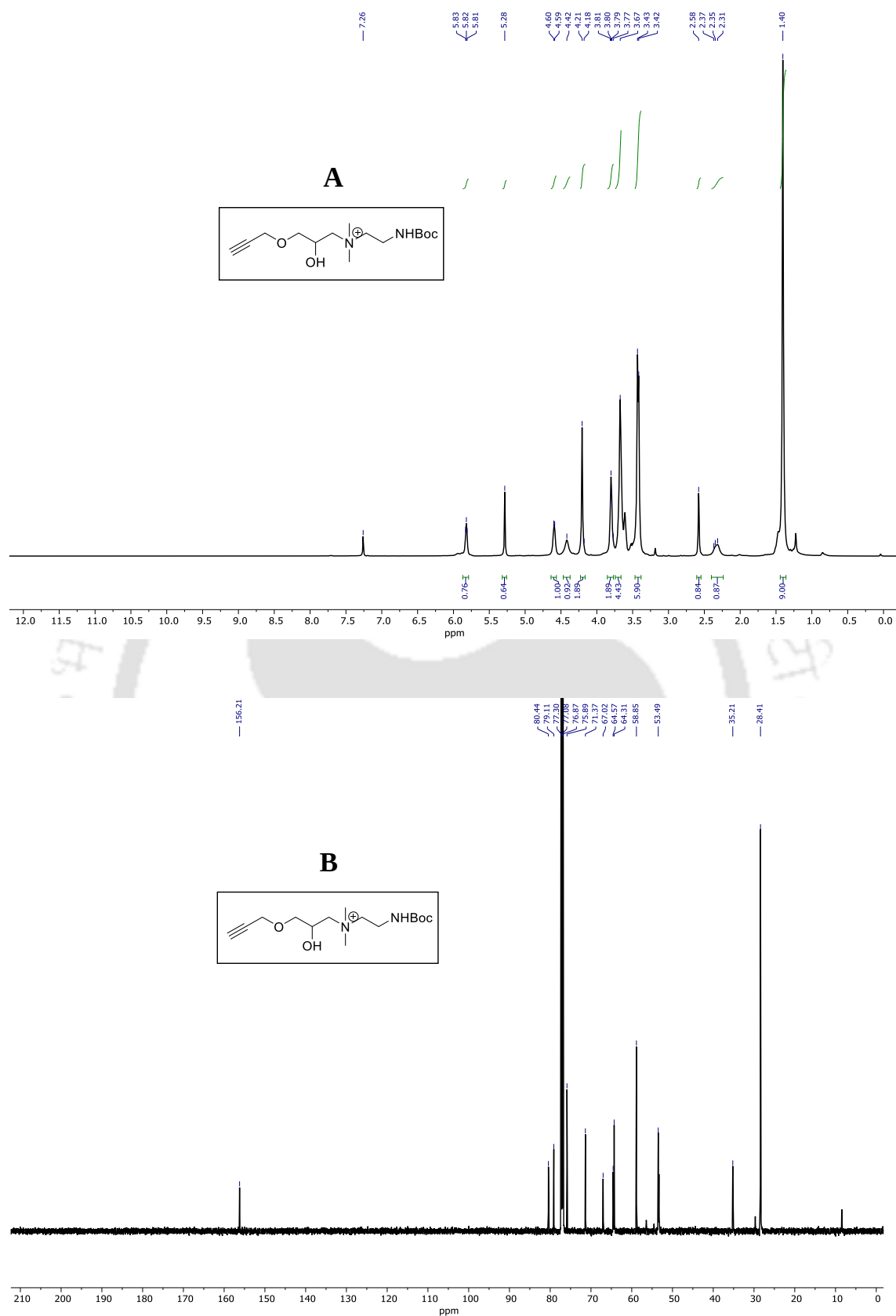


**Figure 2.26.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound **2.10**.



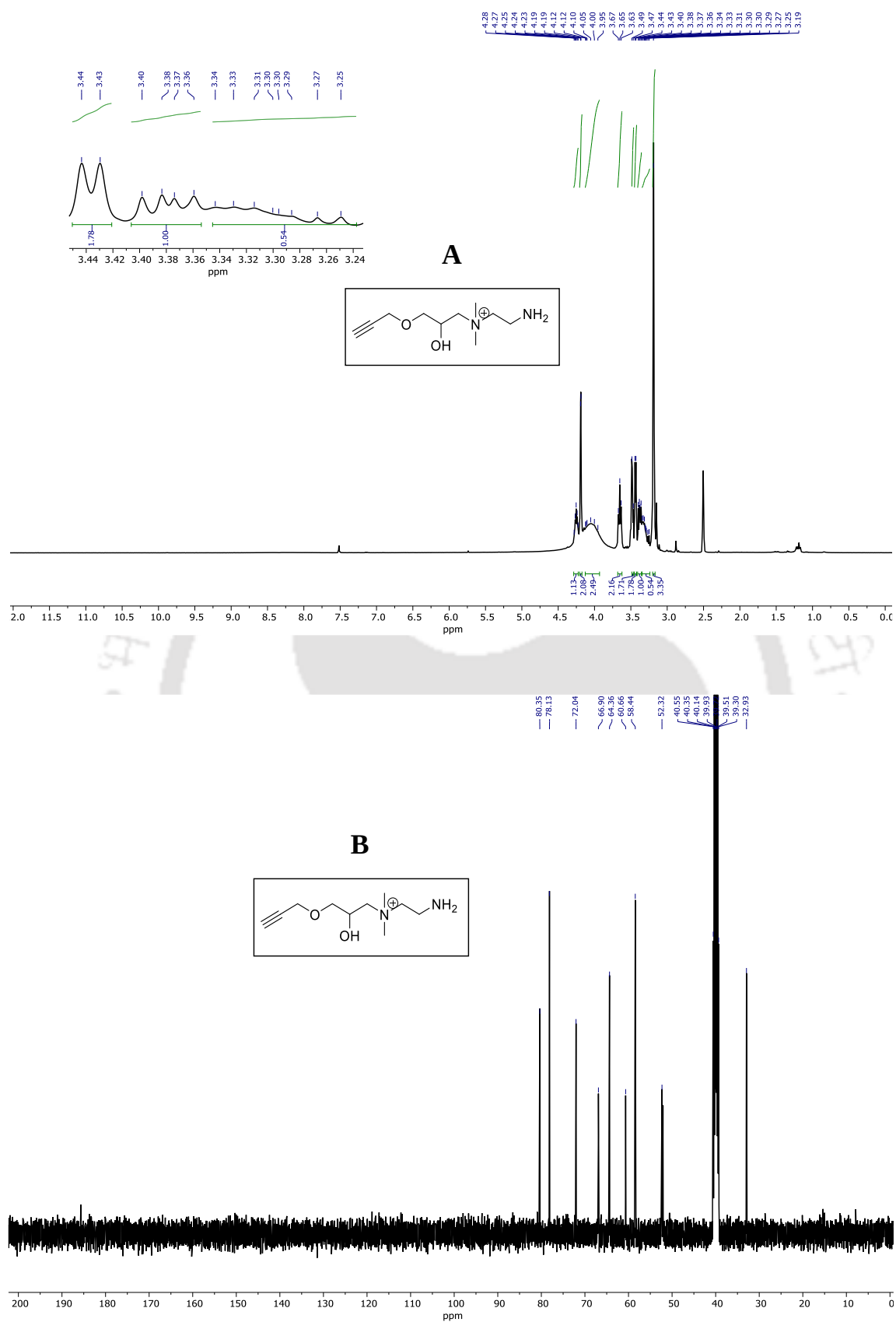


**Figure 2.28.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of compound **2.12**.

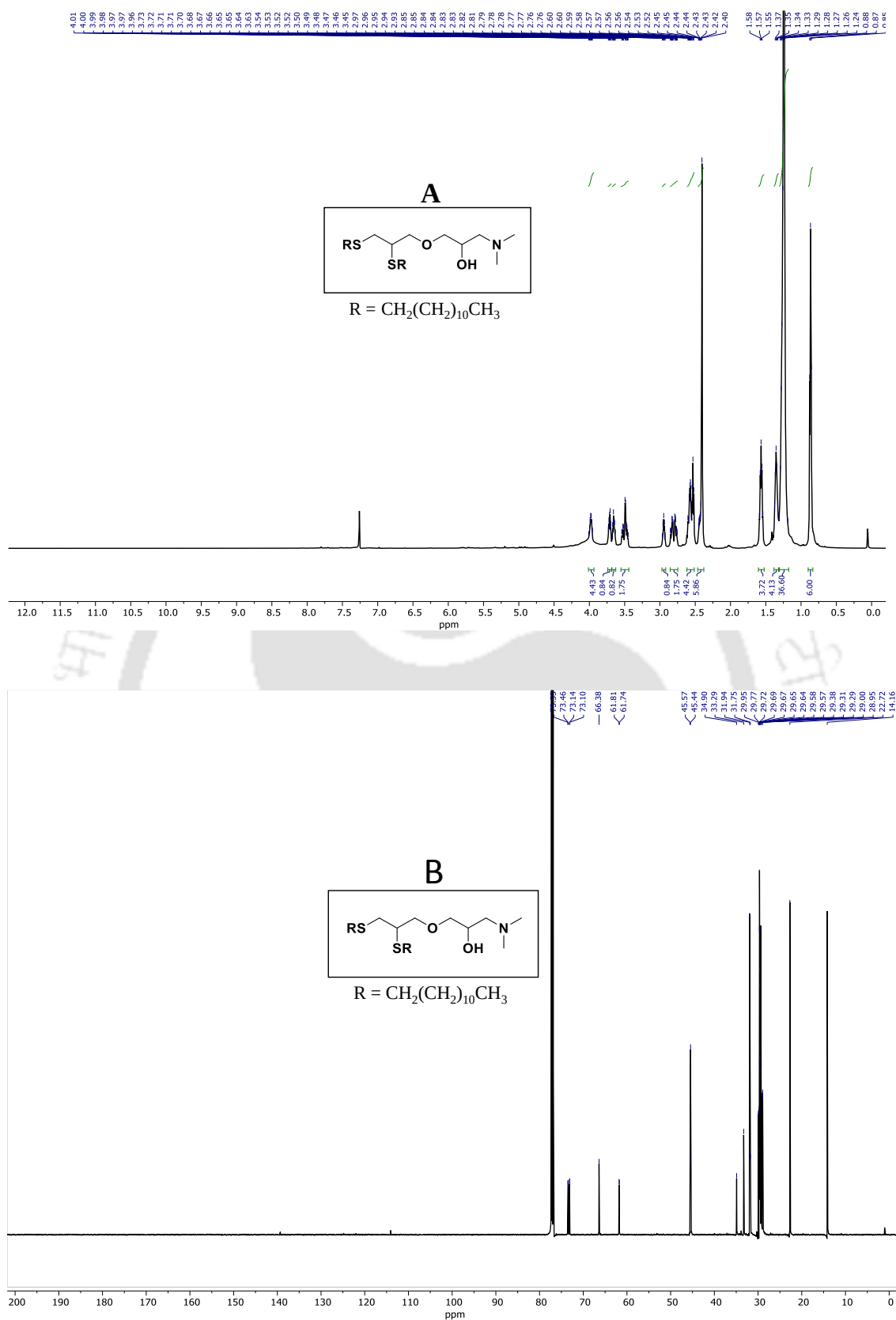


**Figure 2.29.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of compound **2.13**.

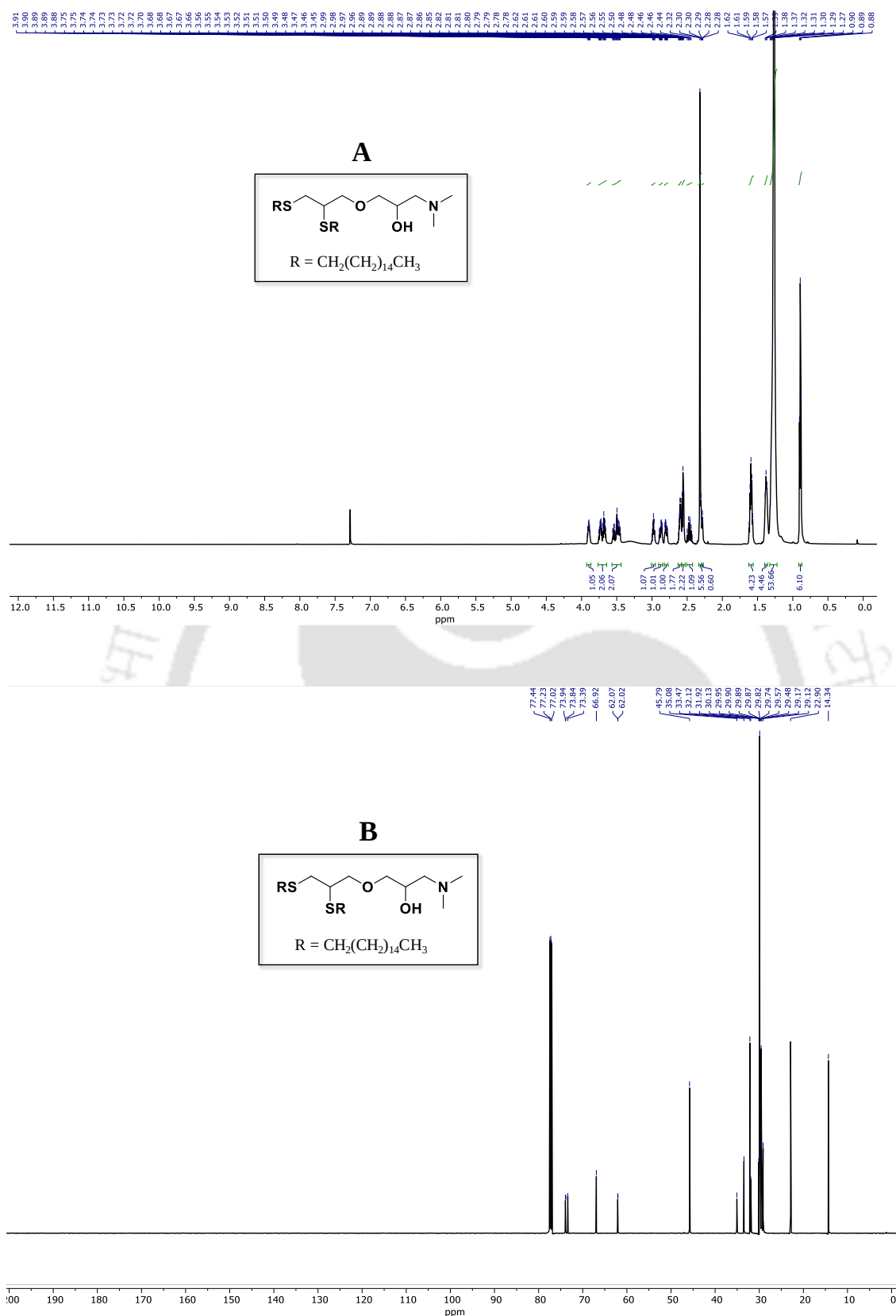




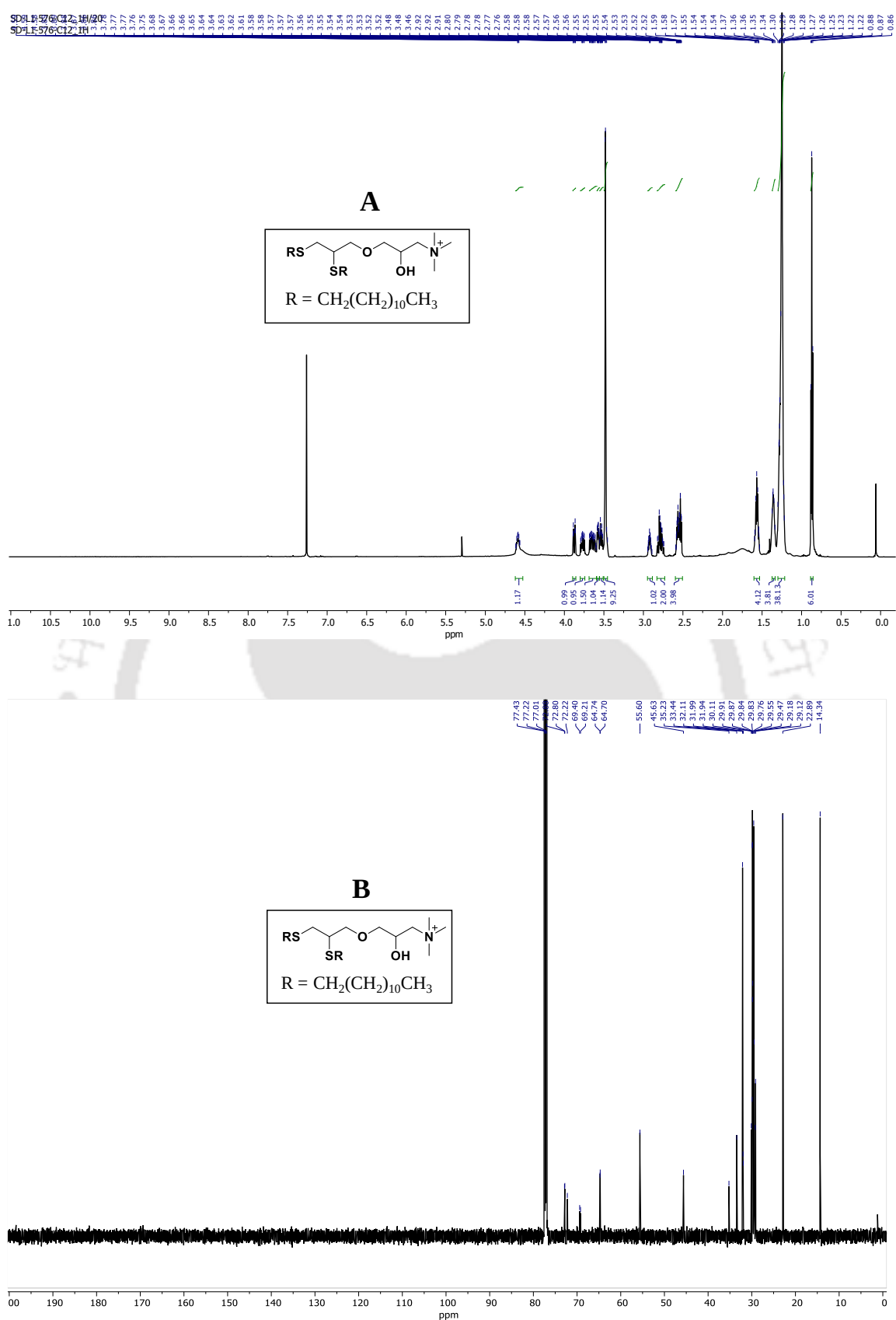
**Figure 2.30.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 2.14.



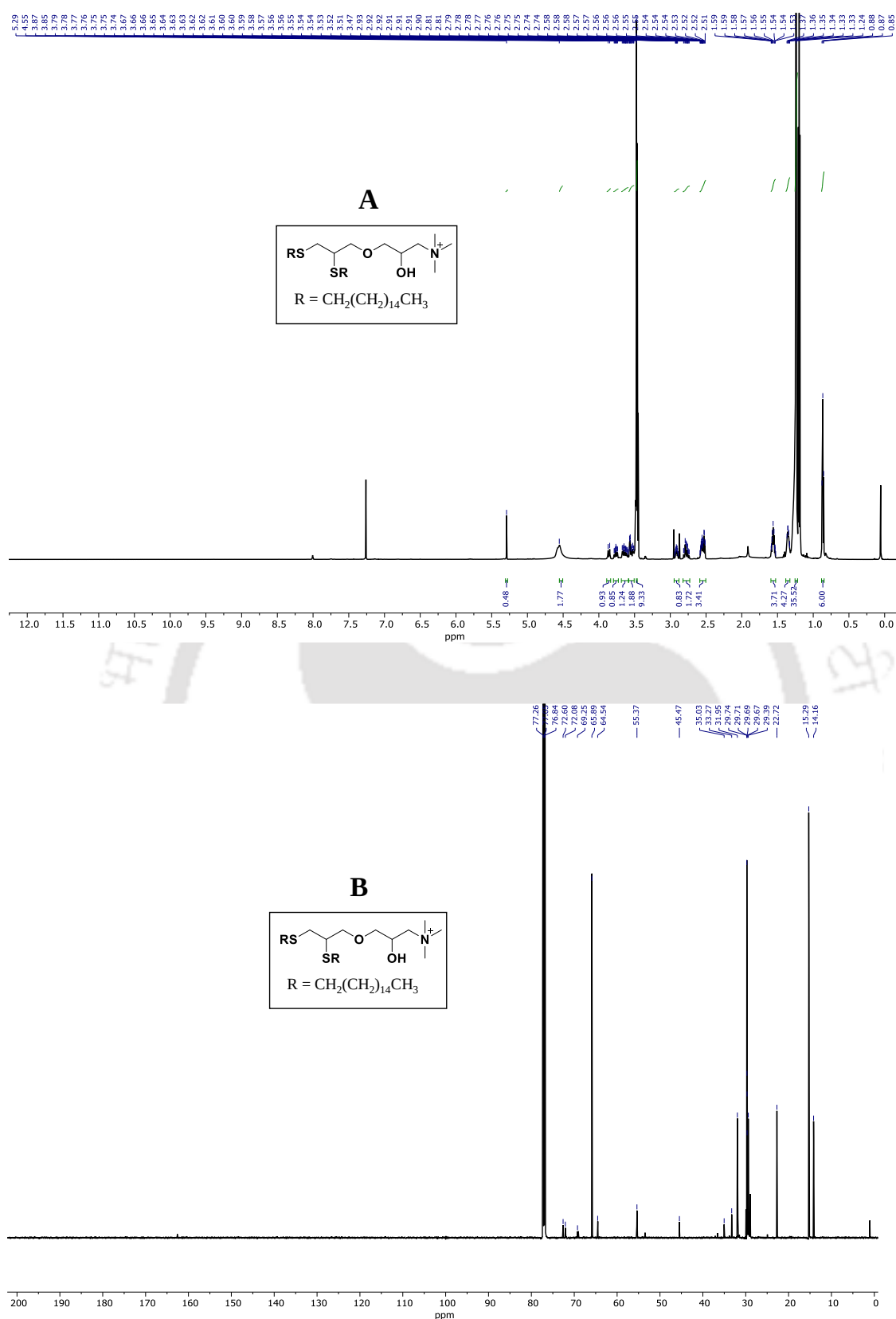
**Figure 2.31.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of lipid **2.15a**.



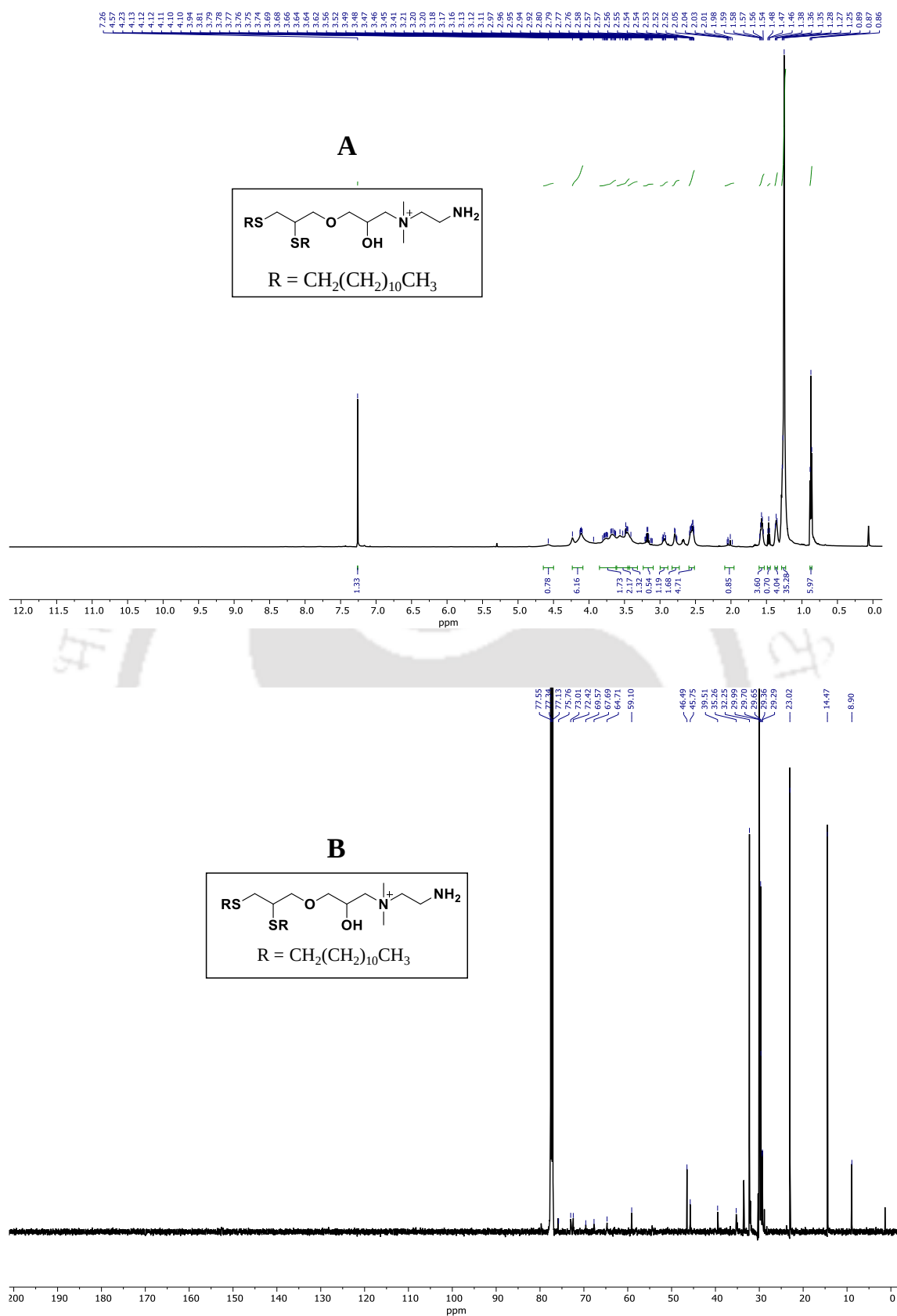
**Figure 2.32.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid **2.15b**.



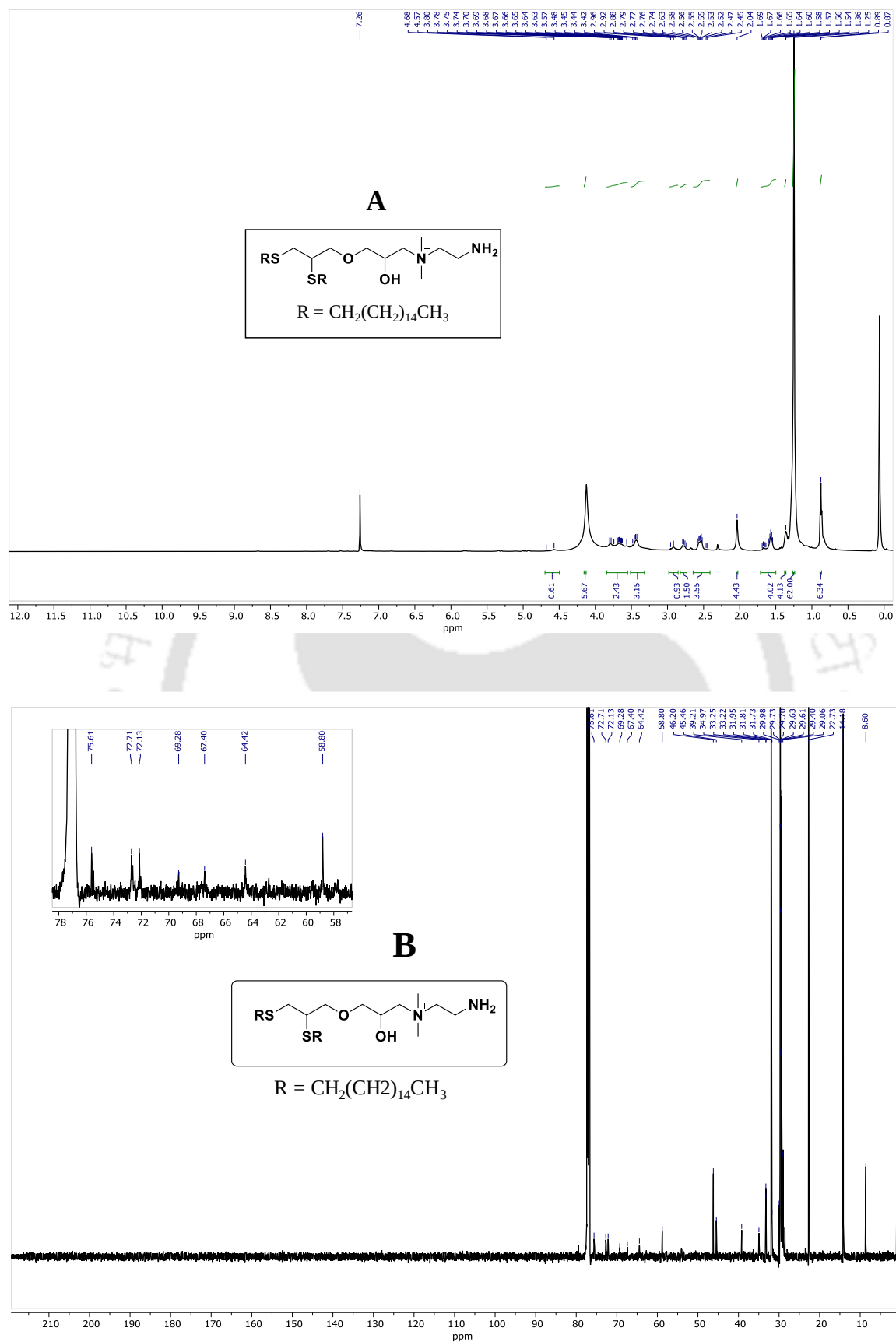
**Figure 2.33.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid **2.16a**.



**Figure 2.34.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid **2.16b**.

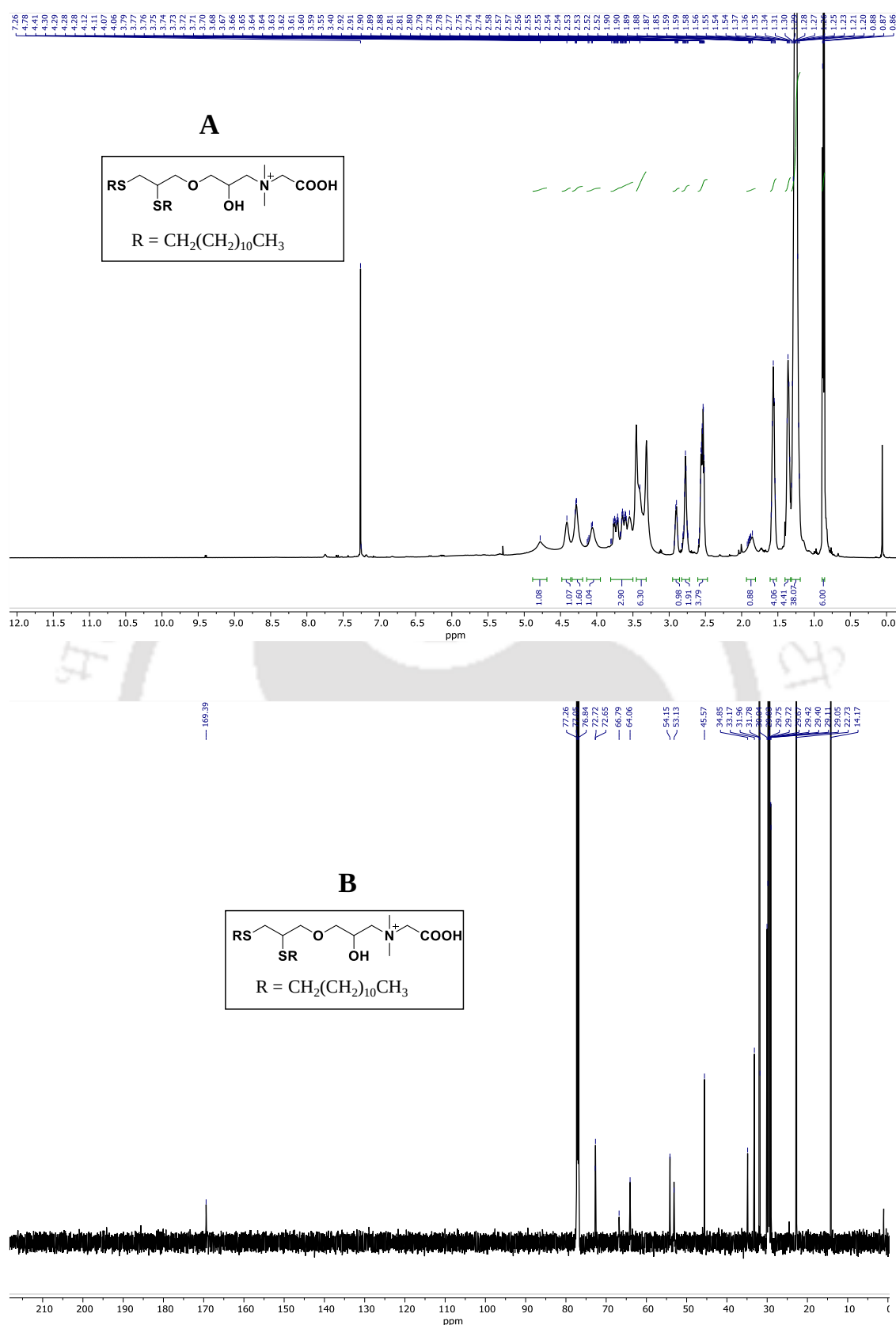


**Figure 2.35.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid **2.17a**.

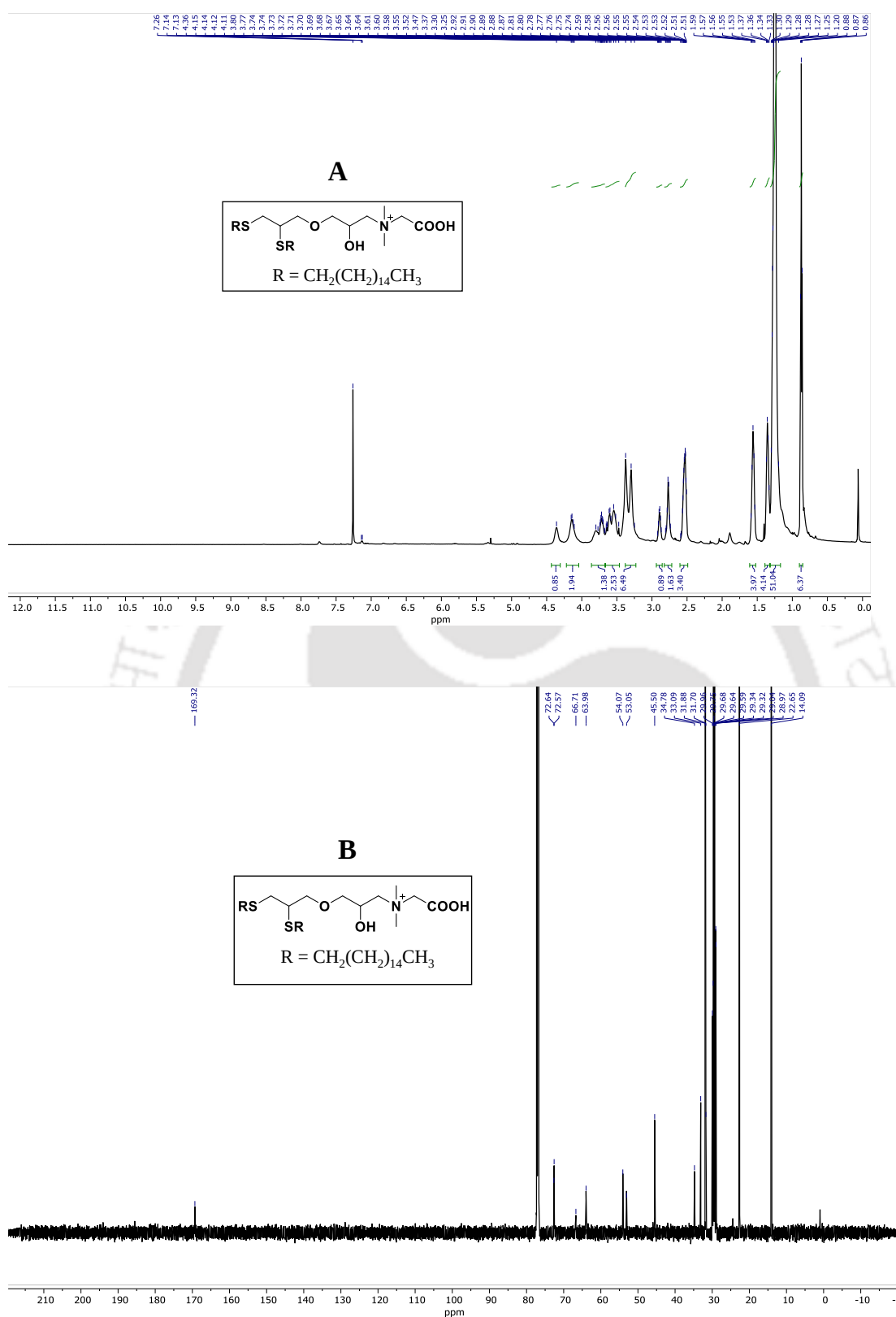


**Figure 2.36.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of lipid **2.17b**.

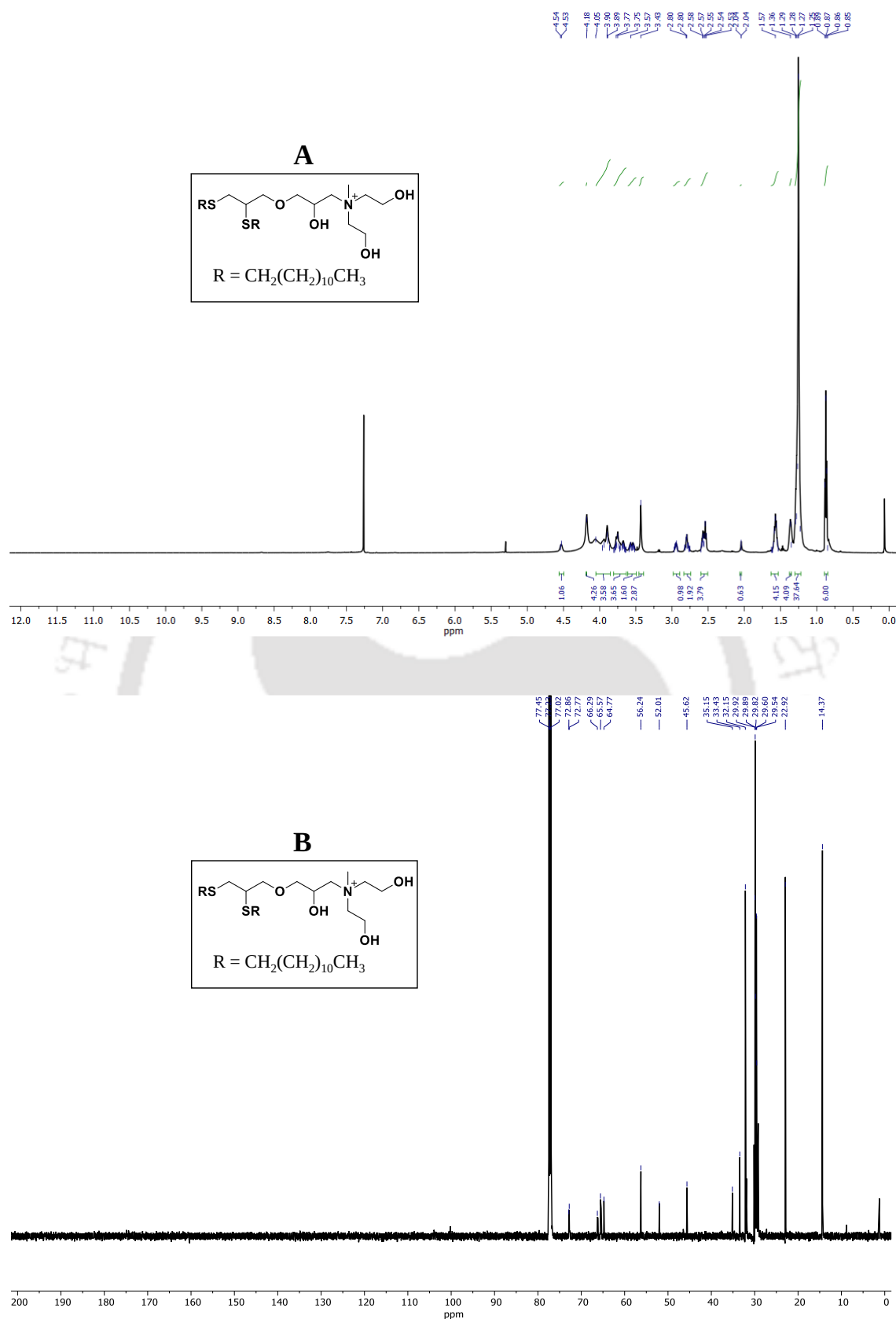




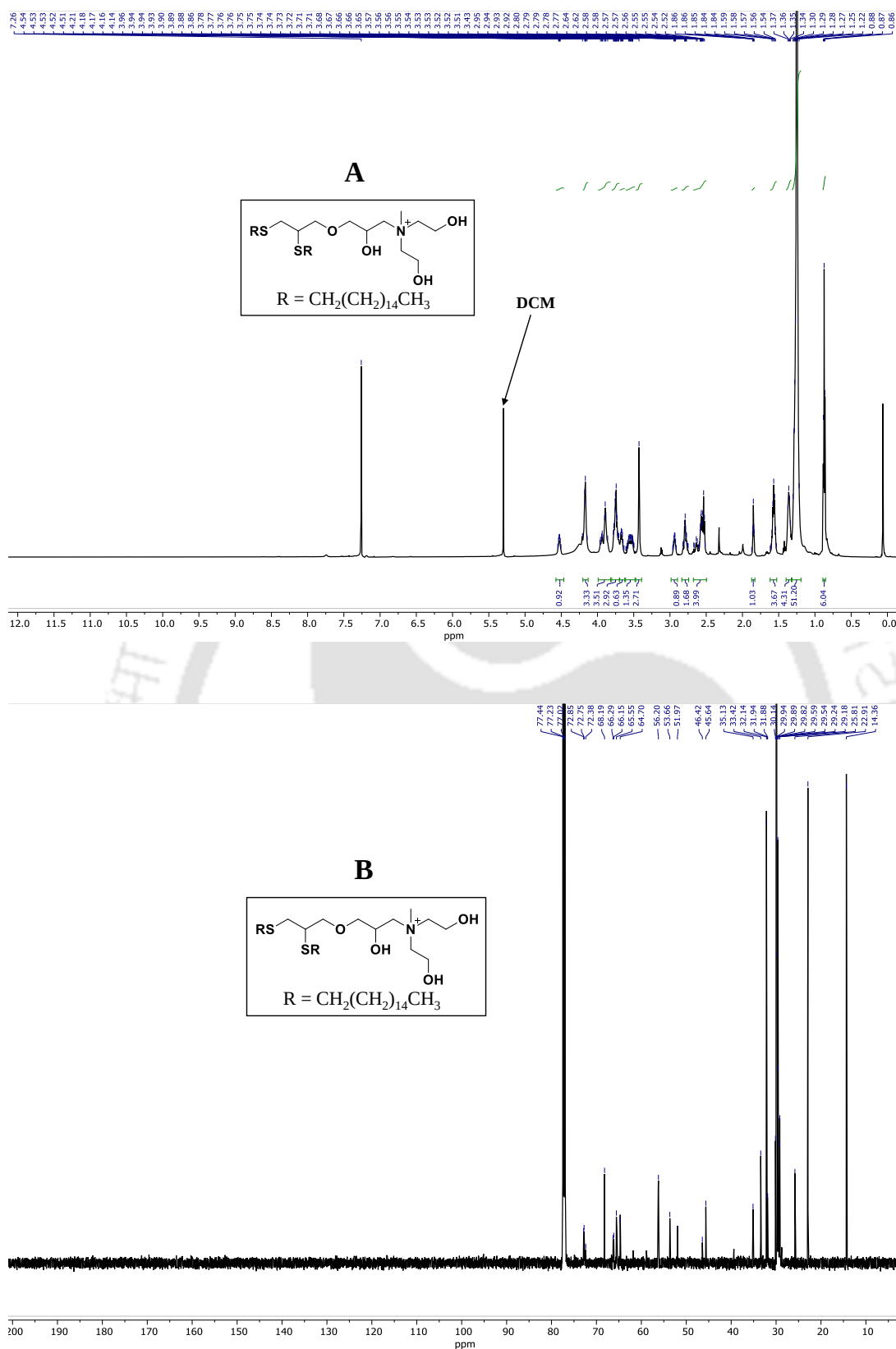
**Figure 2.37.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid **2.18a**.



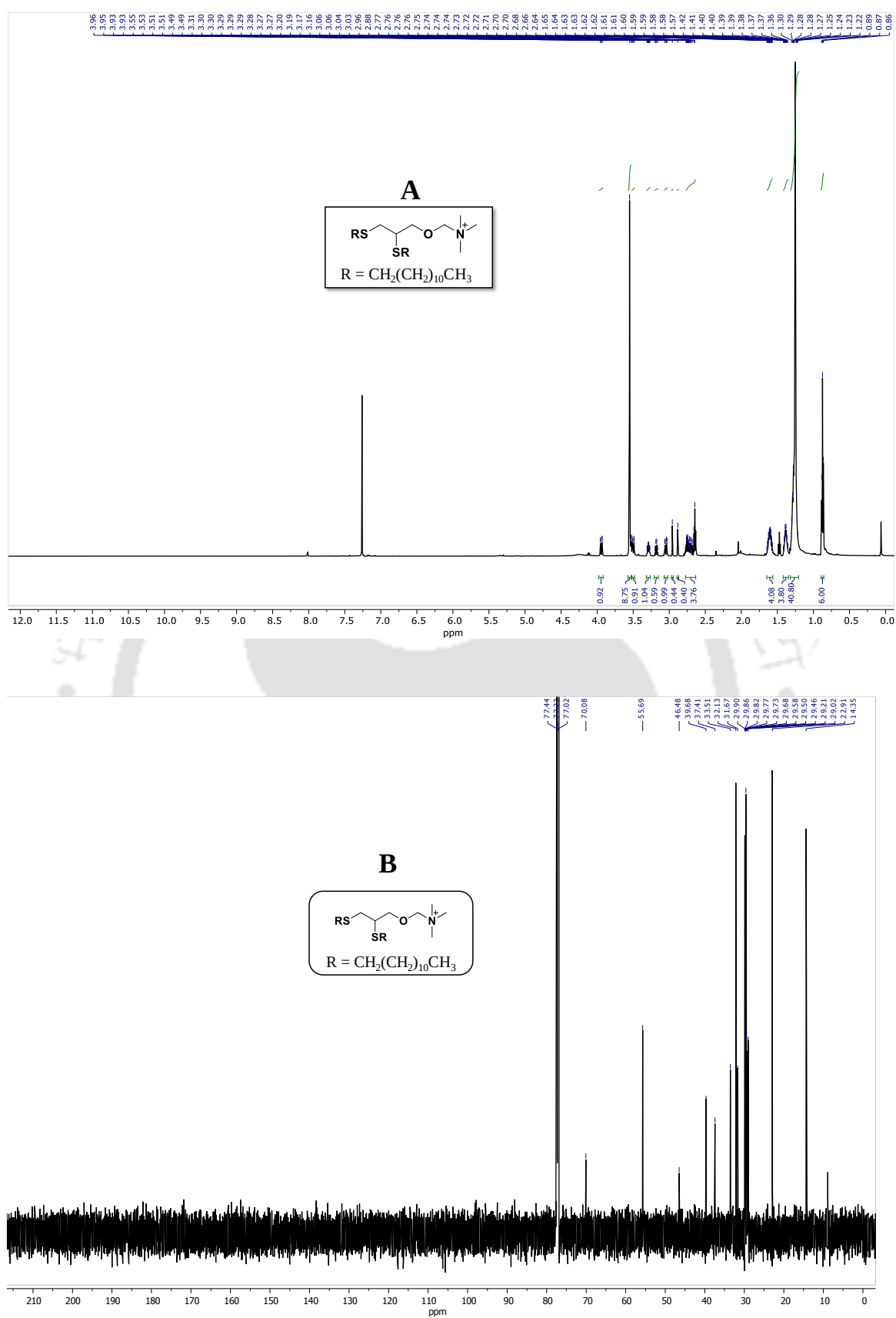
**Figure 2.38.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of lipid **2.18b**.



**Figure 2.39.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of lipid **2.19a**.

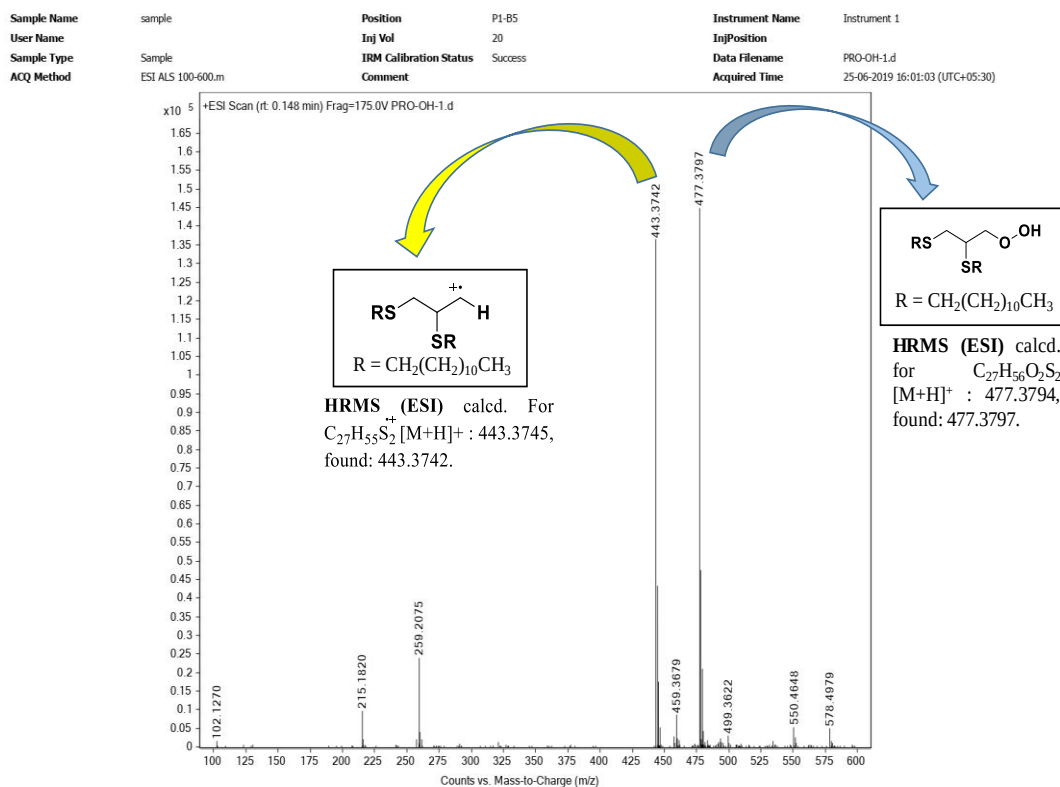
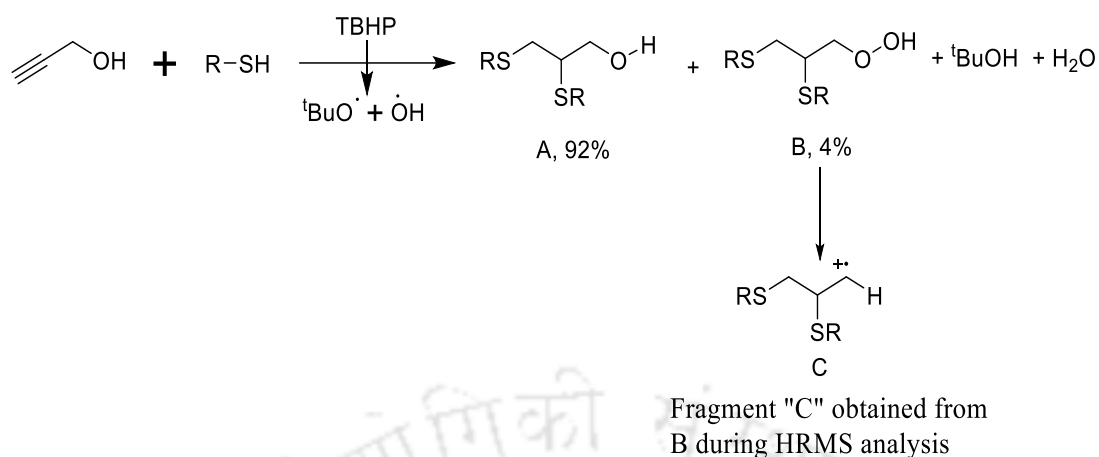


**Figure 2.40.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of lipid **2.19b**.



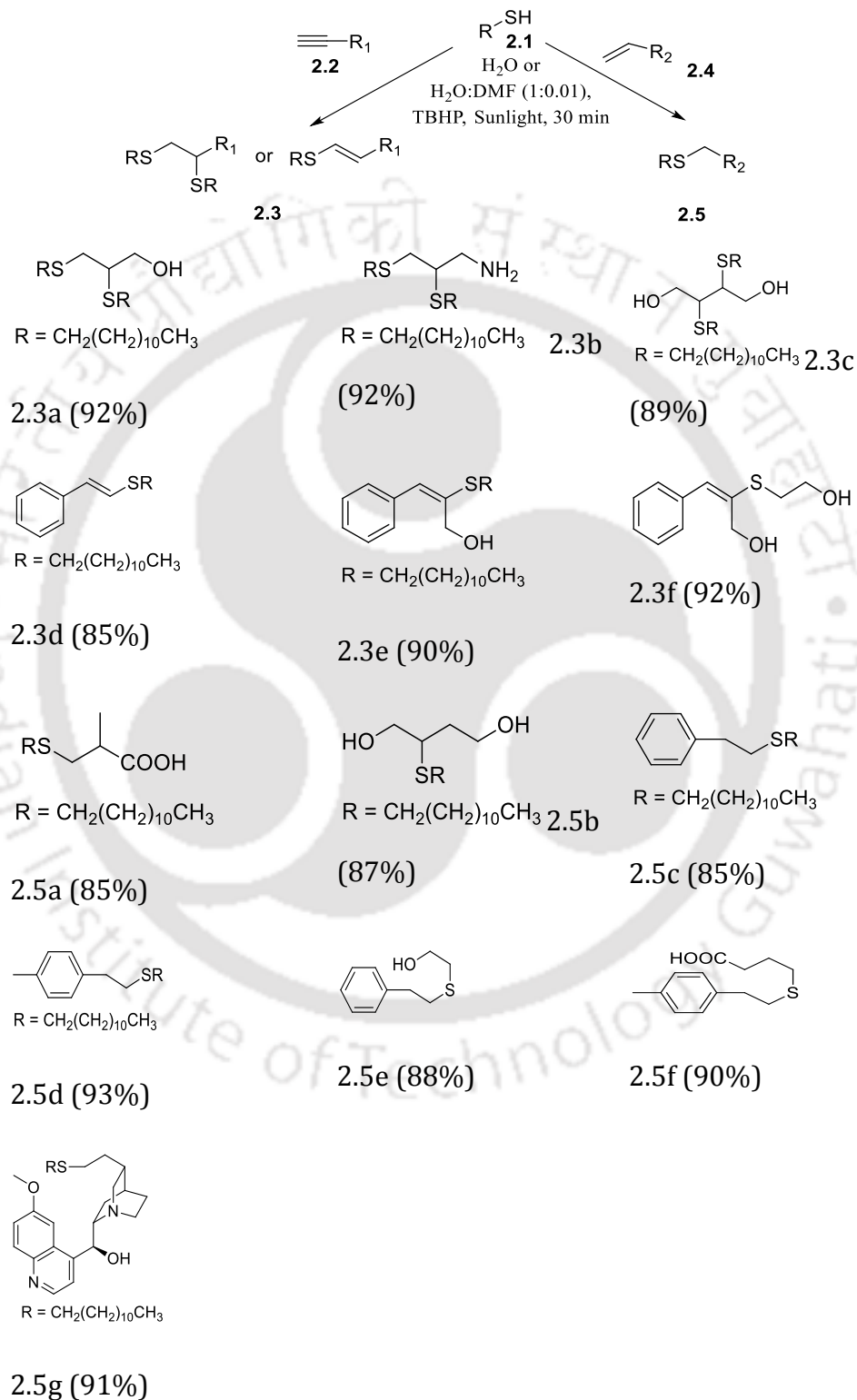
**Figure 2.41.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid **2.20a**.

## 2.4.13. Mass spectrum analysis of the intermediate of thio-yne click reaction



**Figure 2.42.** HRMS analysis of side products of the above reaction indicating the radical-mediated reaction pathway. The compound **B** was isolated, and its HRMS analysis was performed.

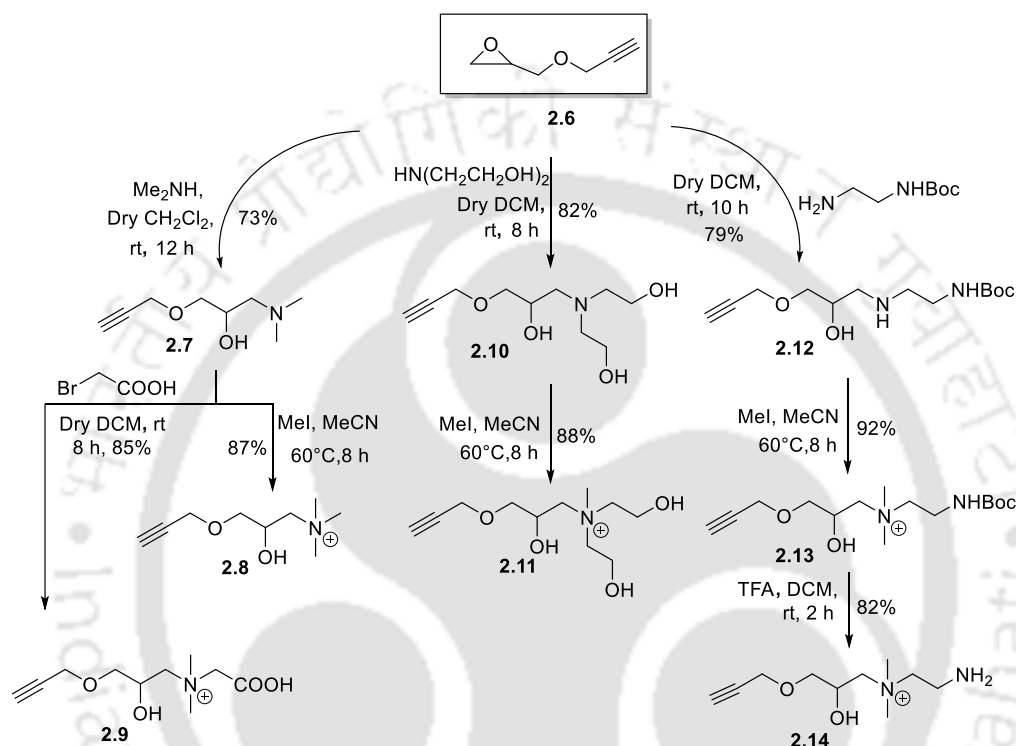
## 2.4.14. Substrate scopes for the synthesis of thioether compounds

**Table 2.3.** Substrate scopes for the synthesis of thioethers via thiol-yne/ene click reactions.



<sup>a</sup>The reactions were performed using 0.046 mmol of **2** or **4** and 1 mmol of **1** in the presence of 0.05 mmol of TBHP and the reactions for compounds **3a-c** were performed in H<sub>2</sub>O (2 mL) and **3d-f** and **5a-g** were performed in H<sub>2</sub>O/DMF (2 mL) solvent mixture (1: 0.01, v/v)

#### 2.4.15. Synthetic route of the lipid headgroups



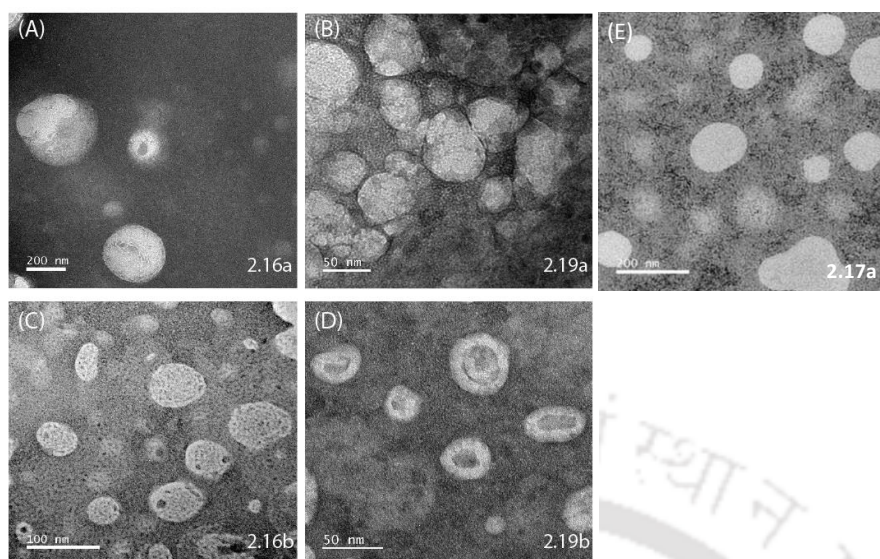
**Scheme 2.1.** Synthetic route to lipid headgroups

#### 2.4.16. Gel retardation assay of the control compound



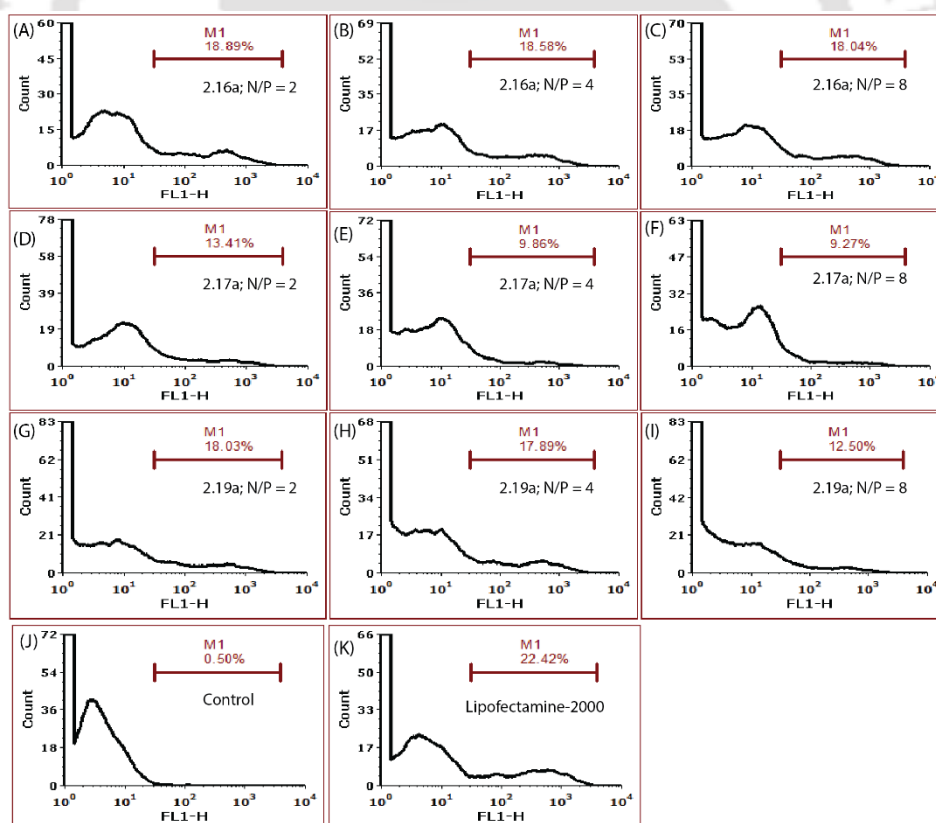
**Figure 2.43.** Electrophoretic gel retardation assay of pMMP7-GFP in the absence and presence of co-liposomes at different N/P ratios (0 to 8). The molar ratio of synthetic lipid (**2.20a**) and PE was 1:1.

### 2.4.17. TEM images of lipoplexes



**Figure 2.44.** TEM images of the lipoplexes of lipids **2.16a** (A), **2.19a** (B), **2.16b** (C) **2.19b** (D) and **2.17a** (E) at N/P ratio of 2 in deionized water. The molar ratio of synthetic lipid and PE was 1:1.

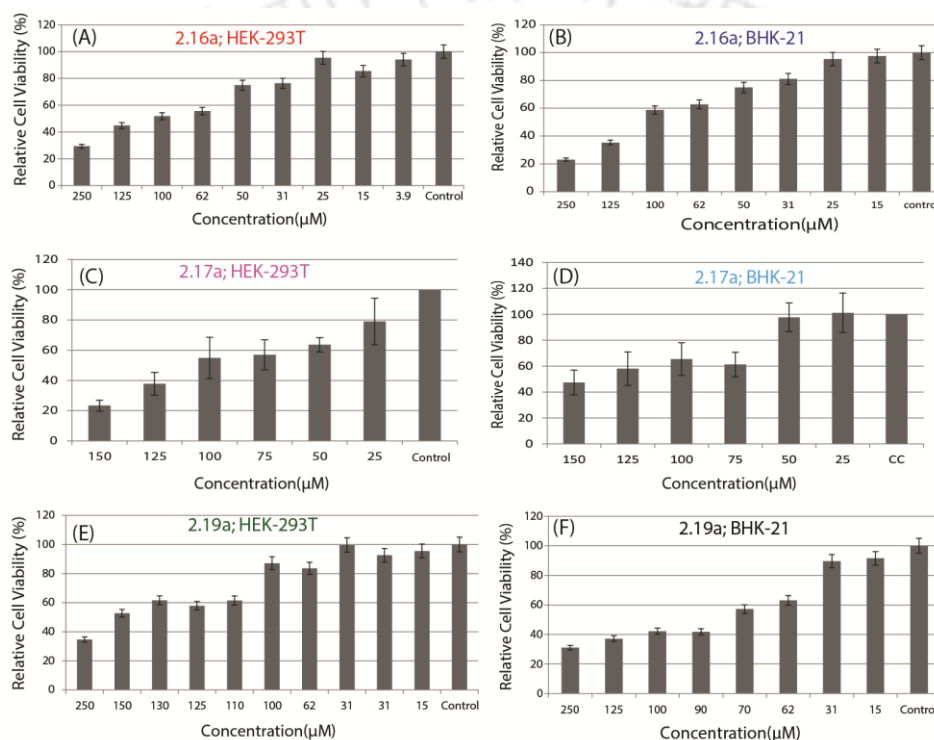
### 2.4.18. FACS analysis for pMMP7-GFP uptake



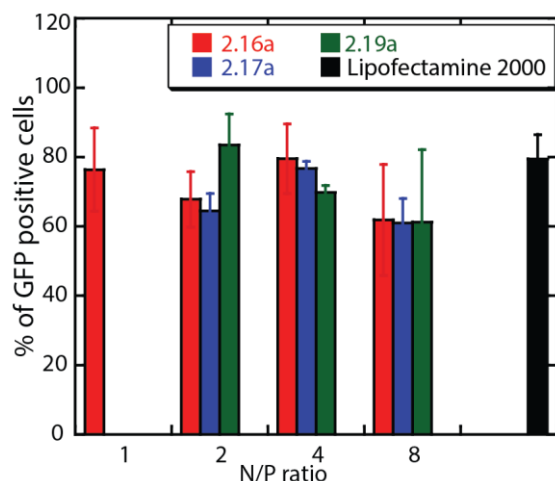
**Figure 2.46.** Analyses of the cellular uptake efficiencies of pMMP7-GFP DNA by the lipoplexes. The uptake efficiencies of the lipids were accessed by measuring the uptake efficiencies of the GFP fluorescence reporter MMP-7 gene. Representative

histograms are shown. The FACS analysis of lipid **2.16a** at N/P ratios of 2 (A), 4 (B) and 8 (C) were measured. The FACS analysis of lipid **2.17a** at N/P ratios of 2 (D), 4 (E) and 8 (F) were measured. The FACS analysis of lipid **2.19a** at N/P ratios of 2 (G), 4 (H) and 8 (I) were measured. The FACS analysis of control (no additive; J) and in the presence of lipofectamine-2000 (K) were also measured. FL1 channel was used to measure green fluorescence. The M1 region is indicated in plots capturing green fluorescence intensity in transfected cells, which is more than auto fluorescence intensity.

#### 2.4.19. Cellular viability of the lipids.



**Figure 2.47.** Concentration-dependent viability of HEK-293T and BHK-21 cells in the absences and presence of the lipids. The MTT assay of lipids **2.16a** (A), **2.17a** (C) and **2.19a** (E) was performed in HEK-293T cells at different concentrations. The concentration-dependent MTT assay of lipids **2.16a** (B), **2.17a** (D) and **2.19a** (F) were also performed in BHK-21 cells



**Figure 2.48.** The MMP7-GFP transfection efficiency (%) of the lipids at different N/P ratio in HEK-293T cells after 24 hours of transfection

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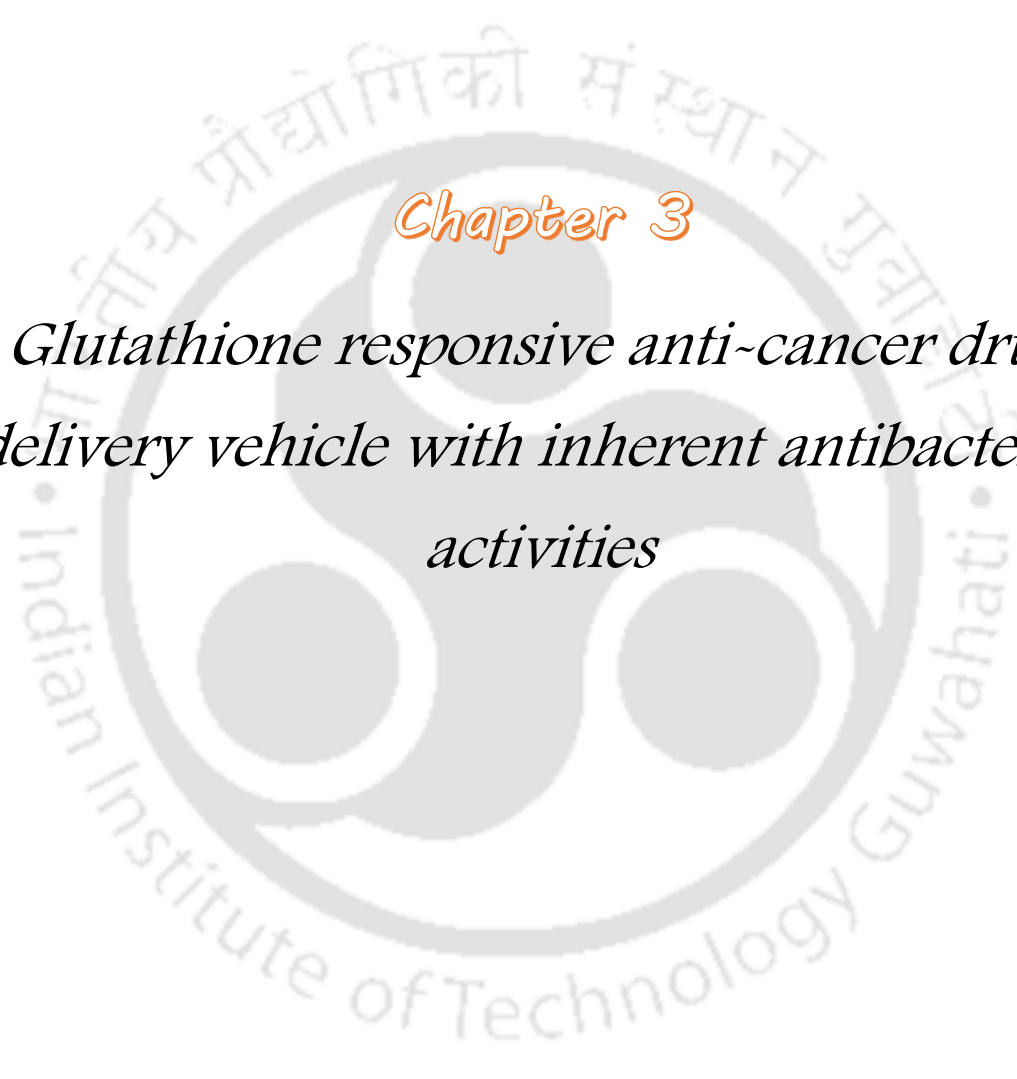
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## Chapter 3

*Glutathione responsive anti-cancer drug  
delivery vehicle with inherent antibacterial  
activities*



### 3.1. Background and objective of present work

The delivery of drug molecules to specific cells or tissues through the blood circulation system is one of the major impediments in pharmaceutical research.<sup>1-3</sup> In this regard, the stimuli-responsive drug delivery strategy has been considered one of the practical approaches in terms of drug efficacy, especially for cancer treatment.<sup>4-7</sup> Significant changes in internal physiological conditions, including the pH, redox potential, protein/enzyme levels, and others of the tumor microenvironment, have been used as stimuli for chemotherapeutic drug delivery applications.<sup>8-9</sup> These stimuli cause a significant change in the chemical structure of the drug carriers due to the cleavage of their specific functional groups or the changes in their physicochemical properties, which instigate the release of encapsulated drug molecules in-or-around diseased cells.<sup>4, 10</sup> GSH-sensitive lipids may provide a practical approach to develop potential drug carriers. Using the thiol-disulfide exchange reaction between GSH and the disulfide bond of the carrier and the difference in GSH concentration between normal cells and cancer cells, a large number of complex polymeric materials have been developed as drug carriers.<sup>11-13</sup> Unfortunately, GSH-sensitive vesicle formulations have not been well explored. Recently, GSH-sensitive disulfide containing phosphatidylcholines (SS-PCs) has been demonstrated.<sup>14</sup> However, this redox-sensitive disulfide-based nanocarrier showed a quick response to S-S bond cleavage in the cellular microenvironment. This high reactivity of GSH towards the S-S bond limits its intracellular uptake and increases the risk of cytotoxic effects.<sup>14-15</sup>

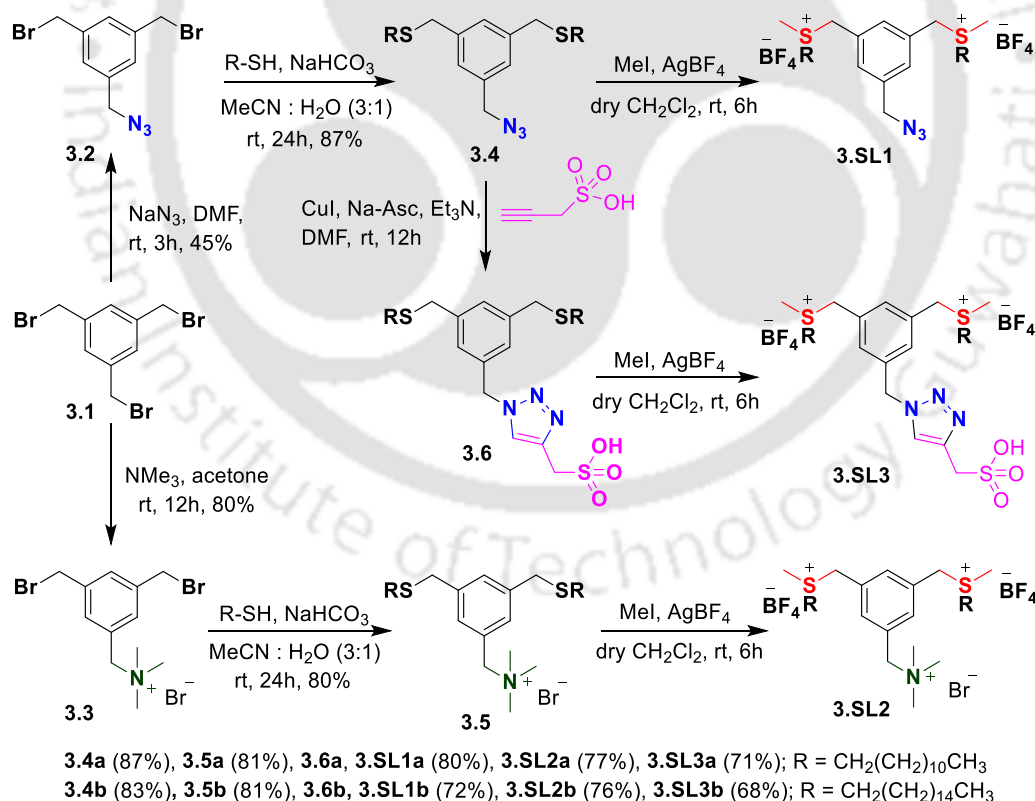
The present study describes the design and presence of several therapeutic agents, including bleomycin and adenosylmethionine. It is reported that sulfonium-containing compounds are less toxic to mammalian cells in comparison with other onium analogs.<sup>16</sup> The higher concentration of GSH is only known to cleave the sulfonium moieties in a controlled manner.<sup>17-18</sup> We postulate that the presence of the sulfonium moiety could improve the cellular uptake efficiency and drug release profile of these lipids in comparison to disulfide-containing lipids. We synthesized a series of novel sulfonium-based lipids, which showed a wide range of favorable biophysical properties such as the formation of aqueous soluble spherical aggregates, high phase transition temperature, and stimuli-responsive properties. Additionally, the potent lipid has lower cytotoxicity, and higher efficiency to

encapsulate, and release the hydrophobic drug Dox to cancer cells. The cellular uptake efficiency in the absence and presence of a GSH inhibitor demonstrated that the intracellular GSH level acts as a stimulus to release the drug molecules into cancer cells. Additional studies revealed that the sulfonium-based lipid itself has moderate antibacterial activity against both Gram-positive and Gram-negative bacteria.

## 3.2. Results and discussions

### 3.2.1. Synthesis of sulfonium derivatives gemini lipids

These modular lipids were synthesized using 1,3,5-tris(bromomethyl)benzene as the core unit. The mono-modification with azide and trimethylamine resulted in the formation of compounds **3.2** and **3.3**, respectively. The nucleophilic addition of aliphatic thiols with compounds **3.2** and **3.3** yielded compounds **3.4** and **3.5**, respectively. The azide-alkyne click reaction of compound **3.4** with propynesulfonic acid produced compound **3.6**.<sup>19</sup> Finally, the S-methylation of compounds **3.4**, **3.5**,

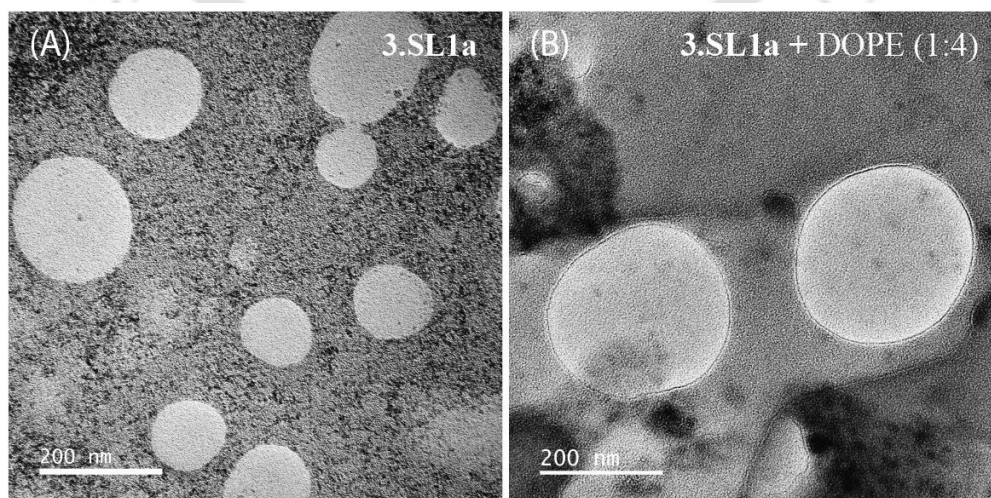


**Scheme 3.1.** Synthetic routes of the sulfonium lipids.

and **3.6** yielded the target compounds **3.SL1**, **3.SL2**, and **3.SL3**, respectively, with good yields (Scheme 3.1). The long alkyl chains (tail lengths of 12 and 16) were associated with sulfonium moieties for all these SL-lipids. The presence of the azide group provides access to install various headgroups. We hypothesize that the nature and inherent properties of these synthetic sulfonium lipids would be quite different from those of natural glycerophospholipids. Hence, it is vital to explore their physicochemical properties.

### 3.2.2. Morphology of the lipid vesicles

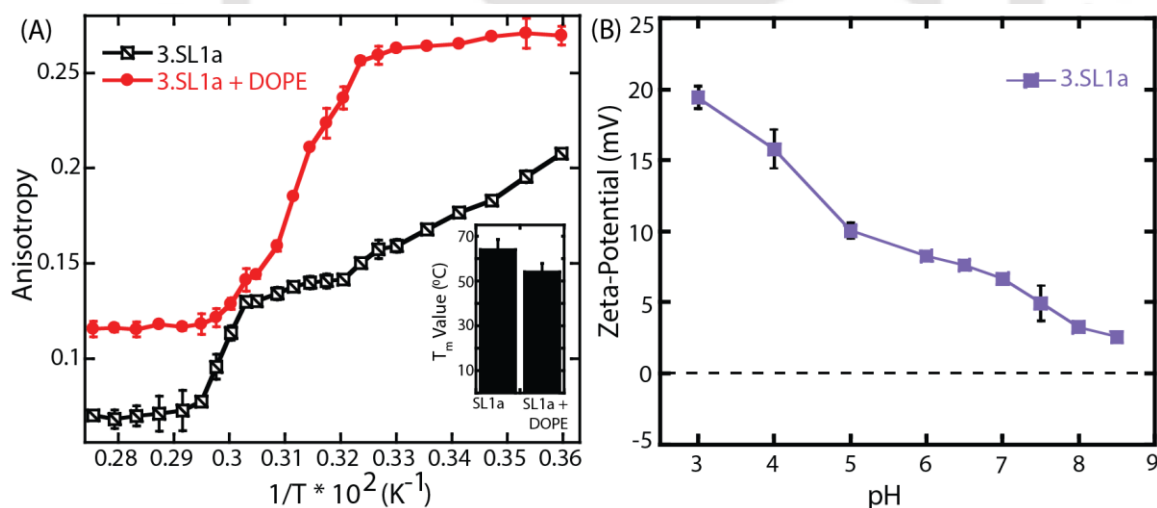
Liposome formation by the sulfonium-based synthesized lipids was verified by transmission electron microscopy (TEM). The images revealed the formation of spherical aggregates of the **3.SL1a** lipid (Figure 3.1A). The TEM analysis also revealed that a mixture of **3.SL1a** and dioleoylphosphatidylethanolamine (DOPE) lipids (1 : 4) forms excellent vesicles (Figure 3.1B), suggesting the competence of the **3.SL1a** lipid in forming vesicles with other phospholipids, which could be beneficial for constructing size-tunable vesicles for specific uses like bio-imaging and drug delivery. The transmission electron microscopy-energy dispersive X-ray spectrometry (TEM-EDX) elemental mappings of sulfur provided direct evidence of the self-assembled structure of the **3.SL1a** lipid (Figure 3.19). However, the other synthetic lipids were less efficient in the formation of any soluble aggregates, which could be due to their higher hydration energies.



**Figure 3.1.** TEM images of NAs of lipids **3.SL1a** (A) and (**3.SL1a** + DOPE) (B).

### 3.2.3. Phase transition temperature measurements

Temperature-dependent steady-state anisotropy of the membrane-embedded 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence probe was employed to determine the Phase transition temperature ( $T_m$ ) values of the membrane bilayer generated from the sulfonium lipids.<sup>20-21</sup> The analysis revealed that the ( $T_m$ ) of the **3.SL1a** lipid was 64 °C (Figure 3.2A). The higher  $T_m$  values demonstrate the higher thermal stability of the vesicles. On the other hand, the  $T_m$  value of a co-liposome (**3.SL1a**:DOPE of 1 : 4) was 54 °C, which is much higher than the phospholipid DOPE ( $T_m = -16$  °C),<sup>22</sup> suggesting that the presence of sulfonium moieties plays a crucial role in their molecular arrangements. The higher  $T_m$  value of the **3.SL1a** vesicles and co-liposome than normal human body temperature (37 °C) also suggests their better drug retention ability in comparison with only DOPE, which indicates their prospective application in thermally triggered drug delivery systems. It is important to mention that the increase in the kinetic energy of the vesicles with the increase in temperature could induce the rearrangement of their aggregation pattern prior to or after their phase transition.



**Figure 3.2.** The steady-state fluorescence anisotropy measurement of 1,6-diphenyl-1,3,5-hexatriene of the lipids within the range of 5–90 °C. The Inset shows the  $T_m$  values of the lipids (A) and surface potential of the vesicles generated from **3.SL1a** lipid at different pH (B).



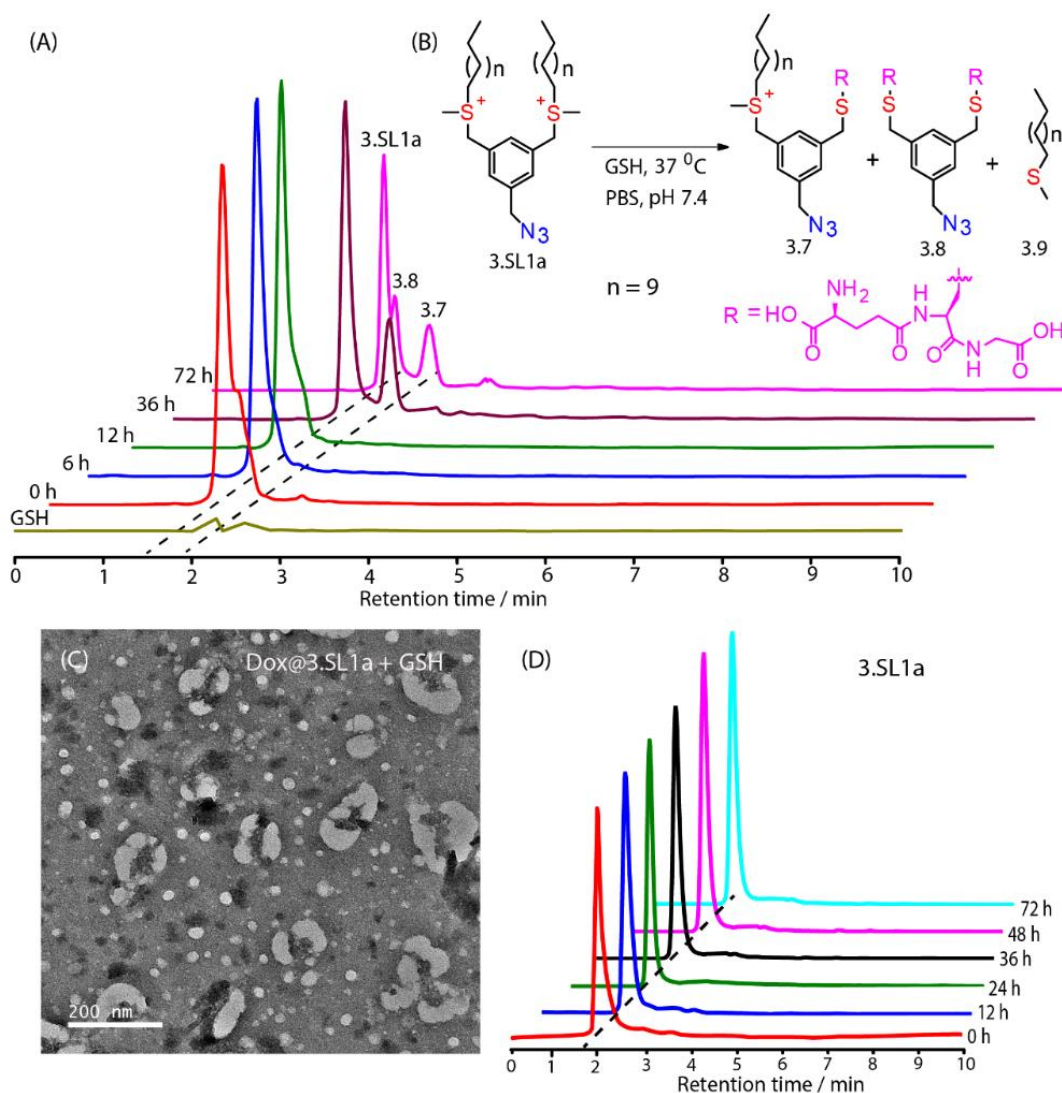
### 3.2.4. Zeta potential and hydrodynamic diameter of the lipid vesicles

The dynamic light scattering (DLS) measurement revealed that the hydrodynamic diameters ( $d_H$ ) of the vesicles were within 200–450 nm, and the pH of the solution had a negligible effect on its  $d_H$  values (Figure 3.20).<sup>21, 23</sup> The zeta-potential measurement at various pH values revealed that the vesicles form a positive surface potential, which could be due to the presence of sulfonium moieties (Figure 3.2B). The decrease in the surface-potential value with the increase in pH suggests that the reduction of sulfonium positive charge could be due to the increase in the solvation number of the negatively charged hydroxyl group.

### 3.2.5. Glutathione mediated degradation of the lipid

To confirm the cleavage of sulfonium moiety of the **3.SL1a** lipid, we performed the HPLC analysis in the absence and presence of GSH (10 mM) in PBS (pH 7.4) at 37 °C (Figure 3.3A). The time-dependent HPLC analysis revealed that the formation of mono- and di-dealkylated intermediates start after 36 and 72 h of incubation in the GSH environment (Figure 3.3A and B). The dealkylations of the **3.SL1a** lipid were scrutinized by mass spectrometry (Figure 3.21 and 3.22)<sup>15</sup> The control experiment showed that the **3.SL1a** lipid is very much stable in the absence of GSH under similar experimental conditions (Figure 3.3D). The TEM and TEM-EDX analysis of the **3.SL1a** lipid in the presence of GSH also showed a significant change in the vesicles' aggregation arrangements, indicating its instability under the reduced environment (Figure 3.3C).



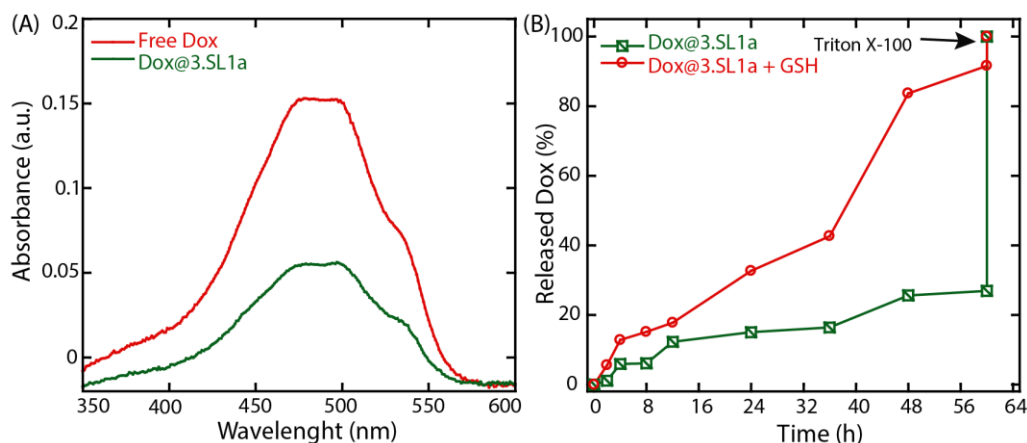


**Figure 3.3.** The HPLC traces for the dealkylation of lipid **3.SL1a** in the presence of GSH (10 mM) at different time intervals (A). Schematic representation of the dealkylation strategy (B). The TEM image of the **3.SL1a** lipid in the presence of GSH (10 mmol) after 36 h of incubation (C). The HPLC traces for the dealkylation of lipid **3.SL1a** in the absence of GSH at different time intervals (D).

### 3.2.6. Drug loading and release efficiency of vesicle of 3.SL1a lipid

The stimuli-responsive dealkylation-mediated deformation of vesicles of the **3.SL1a** lipid prompted us to investigate its drug encapsulation and release profiles (Figure 3.4A). The **3.SL1a** lipid showed adequate loading capacity (33% at pH 7.4, based on mole fraction) for the hydrophobic anticancer drug doxorubicine (Dox). This adequate drug loading capacity of the **3.SL1a** lipid due to the hydrophobic Dox molecules entrap in the hydrophobic bilayer core of liposome via hydrophobic

interaction. Fluorescence studies revealed that 27% and 88% of Dox were released after 60 h in the absence and presence of GSH, respectively, at 37 °C (Figure 3.4B). The release profile also revealed a drastic difference after 36 h, suggesting the possibility of the destabilization of the **3.SL1a** lipid assembly in the presence of GSH.



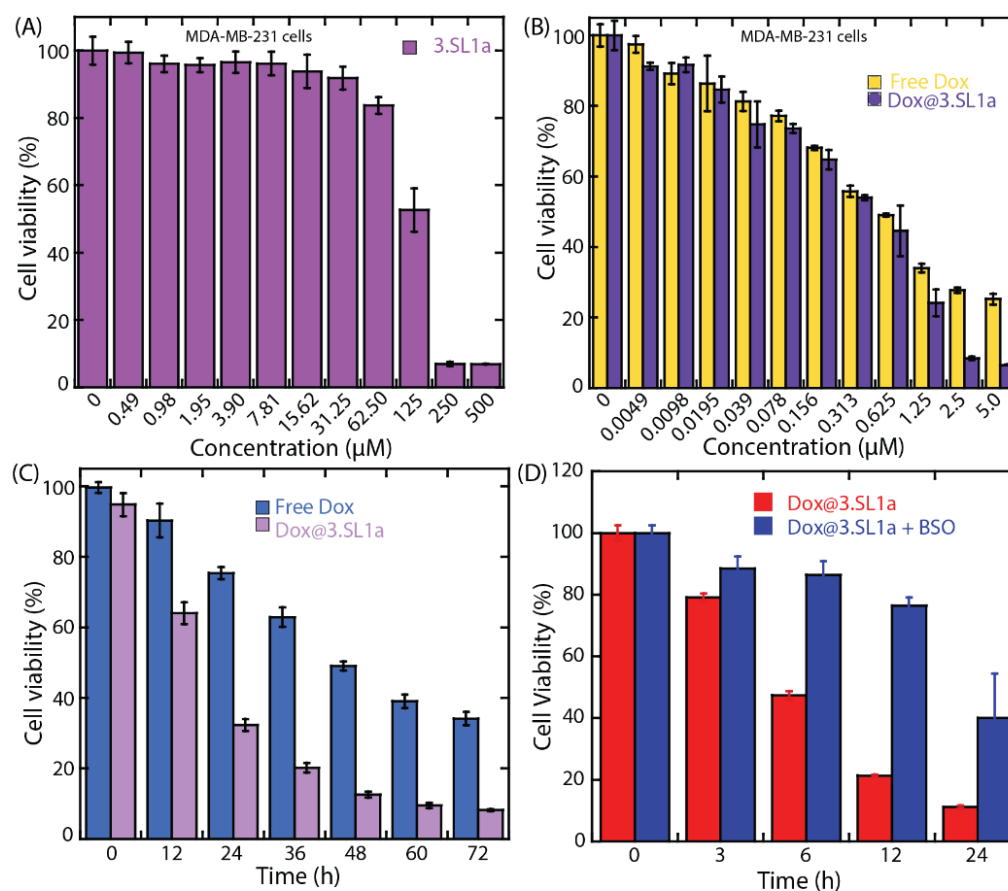
**Figure 3.4.** UV-Vis spectra of free Dox, Dox-encapsulated vesicles of **3.SL1a** lipid at pH 7.4. Dox release profile in the absence and presence of GSH at pH 7.4 (B).

### 3.2.7. Cell viability and cellular uptake analysis

#### 3.2.7.1. Cell Viability

The MTT-based viability assay showed that the **3.SL1a** lipid had a lower cytotoxic effect against MDA-MB-231 (human triple-negative breast cancer;  $IC_{50}$  = 125  $\mu$ M) and BJ (human foreskin fibroblasts;  $IC_{50}$  = 166  $\mu$ M) cell lines, suggesting its relevance to drug delivery applications (Figure 3.5A and 3.23)<sup>24-25</sup> The MTT assay in the presence of free Dox and Dox encapsulated **3.SL1a** lipid (Dox@**3.SL1a**) revealed that the  $IC_{50}$  values were 0.49 and 0.36  $\mu$ M (with respect to the effective Dox concentration), respectively, in the MDA-MB-231 cell line, which support the Dox encapsulation and release efficiencies of the **3.SL1a** lipid (Figure 3.5B) The  $IC_{50}$  value of Dox@**3.SL1a** treated BJ cells was 1.47  $\mu$ M (with respect to the effective Dox concentration; (Figure 3.23) The higher concentration of GSH in MDA-MB-231 cells is attributed to the cleavage of sulfonium lipids, resulting in a much lower  $IC_{50}$  value of Dox@**3.SL1a**.<sup>26</sup> The final concentration of Dox was kept the same for both the MTT assays in the presence of free Dox and Dox@**3.SL1a**. The time-dependent MTT assay showed that the rate of cell death was faster for Dox@**3.SL1a**, indicating its superior

uptake and release of Dox molecules, which is one of the rudiments of a potential drug carrier (Figure 3.5C).

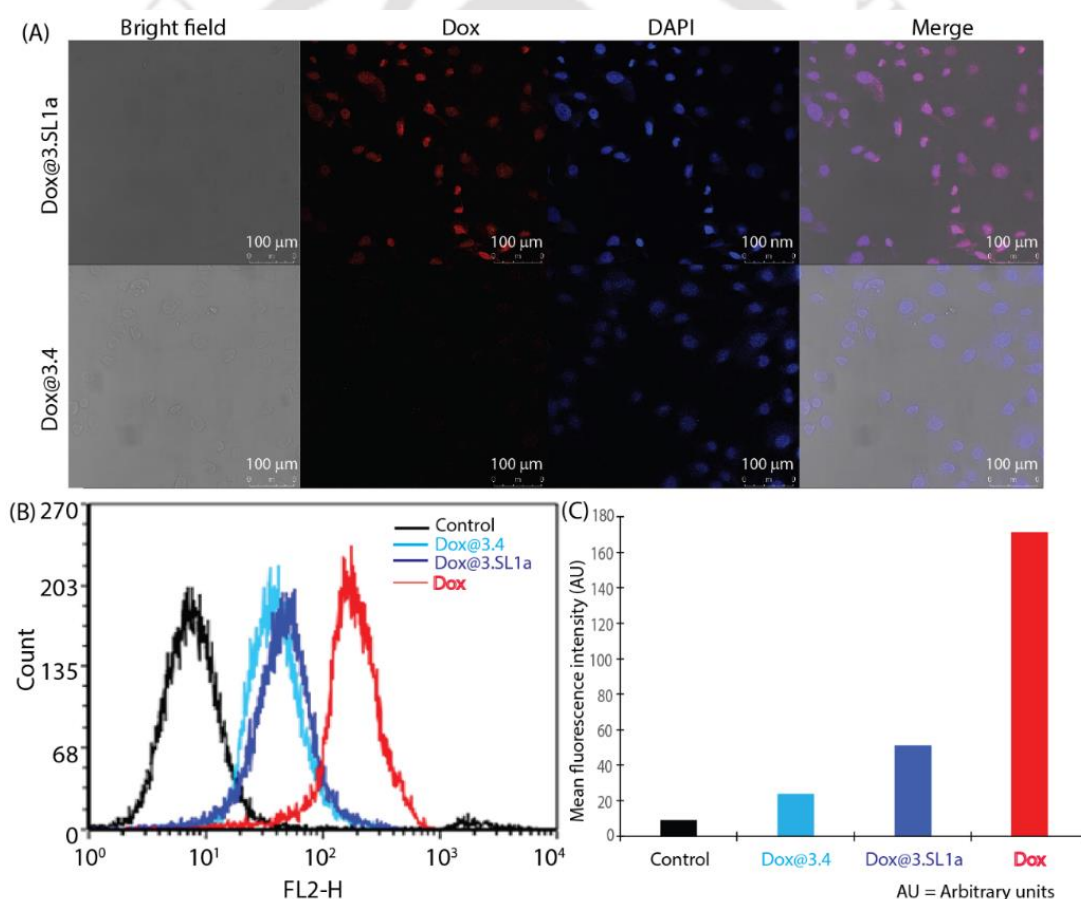


**Figure 3.5.** Viability of MDA-MB-231 cells in the presence of **3.SL1a** (A), free Dox, and Dox@**3.SL1a** were measured by MTT assay after 48 hours of compound treatment. (B) at different concentrations. Time-dependent viabilities of MDA-MB-231 cells in the presence of free Dox and Dox@**3.SL1a** at 0.36 and 0.49  $\mu$ M, respectively (C). Viabilities of the Dox@**3.SL1a** (0.49  $\mu$ M) treated MDA-MB-231 cells in the absence and presence of GSH inhibitor, BSO (1 mM) (D). MTT assay after 48 hours of compound treatment.

To further investigate that the GSH promotes Dox release efficiency from Dox@**3.SL1a** under the cellular environment, the MTT assay was performed with and without a GSH inhibitor, buthionine sulfoximine (BSO).<sup>15</sup> The result showed that the cell viability increased in the BSO-treated cells, which could be due to the inhibition of GSH activity (Figure 3.5D).<sup>15</sup>

### 3.2.7.2. Cellular uptake

The cellular uptake efficiency of the **3.SL1a** lipid was further investigated using a confocal laser scanning microscope (CLSM).<sup>27-28</sup> The microscopy images of the MDA-MB-231 cells revealed a higher Dox delivery efficiency of the **3.SL1a** lipid in comparison with lipid **3.4a** (Figure 3.6A). The long alkyl chains of the neutral lipid **4a** were associated with only thioether moieties. To exclude the probability that the fluorescence signal originated due to the adherence of Dox@**3.SL1a** or Dox@**3.4a** to the cell surface, the flow cytometry analysis was performed using the inherent fluorescence signal of Dox molecules (FL2-H channel).



**Figure 3.6.** CLSM images of the MDA-MB-231 cells treated with Dox@**3.SL1a** and Dox@**3.4a** (control lipid without the sulfonium moieties) for 8 h. Phase contrast, Red channel (Dox), blue channel (DAPI), merge of phase contrast, red and blue channels illustrate the Dox delivery. Scale bar 200 nm. Concentration = 0.49 μM. Flow cytometry histogram profiles of MDA-MB-231 cells after 8 h of the treatment with Dox@**3.4a** and Dox@**3.SL1a** and their mean fluorescence intensity. The inherent

fluorescence signal of Dox molecules was used for the measurement (FL2-H channel). Only cells were used as control. Concentration = 0.49  $\mu\text{M}$ .

The result showed that Dox@**3.SL1a** has a higher cellular uptake efficacy than Dox@**3.4a** in MDA-MB-231 cells (Figure 3.6B), which could be due to the superior electrostatic interaction of the positively charged sulfonium moieties with the negatively charged phosphatidylserine (PS) lipid present in higher abundance on the outer surface of the cancer cells.<sup>29-31</sup> This directed electrostatic interaction could also help the cationic sulfonium lipids to reduce the off-target side effects of the Dox itself. The higher cellular uptake of free Dox could be due to its diffusion-mediated delivery to the cells, whereas liposome-mediated Dox delivery generally follows the endocytosis process (Figure 3.6C).<sup>32-34</sup>

### 3.2.8. Antibacterial study

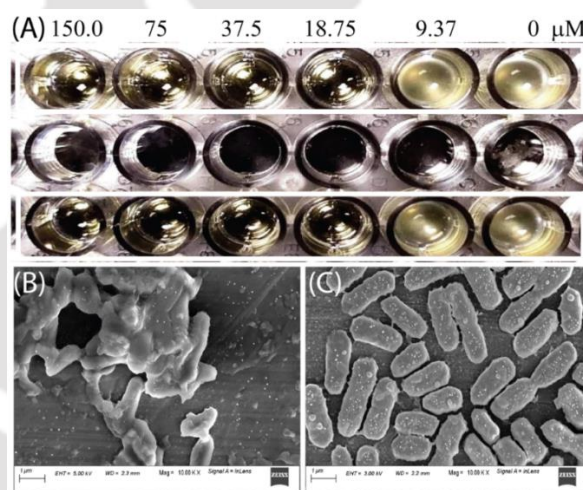
The presence of sulfonium moieties and lower cytotoxicity against mammalian cell lines endorsed us to investigate the antibacterial activities of the **3.SL1a** lipid.<sup>16</sup> The antibacterial activity of the **3.SL1a** lipid was tested against both Gram-negative (*E. coli*; MTCC-1687) and Gram-positive (*S. aureus*; MTCC-96) bacteria, and the minimum inhibitory concentration (MIC) values were  $32 \pm 2$  and  $14 \pm 1$   $\mu\text{M}$ , respectively (Table 3.1, Figure 3.7A and Figure 3.24A). For further investigation, the antibacterial activities were also studied in the presence of free Dox and Dox@**3.SL1a**. Interestingly, the free Dox exhibits much lower antibacterial activities (MIC values  $>72$  and  $36$   $\mu\text{M}$ ) for *E. coli* and *S. aureus*, respectively.<sup>35</sup> On the other hand, the MIC values were 0.19 and 0.10  $\mu\text{M}$  for *E. coli* and *S. aureus*, respectively (with respect to the effective Dox concentration; (Table 3.1), in the presence of Dox@**3.SL1a**. The field emission scanning electron microscopy (FESEM) analysis showed a noticeable loss of cell shape and integrity in the presence of **3.SL1a** and Dox@**3.SL1a** (Figure 3.7B,C and Figure 3.24B,C). Overall, the antimicrobial studies revealed that the ammonium-free drug carrier itself exhibits antibacterial activities. Recent studies revealed that the peptidoglycan of the bacterial cell wall promotes the invasiveness of breast cancer cells by upregulating the Toll-like receptor 2 expressions.<sup>36</sup> Gram-negative bacteria like *E. coli* and *B. fragilis* are also known to produce colorectal cancer-promoting toxins.<sup>37</sup> Therefore,



the inherent antibacterial activity of the sulfonium lipid, along with its anticancer drug delivery efficacy, represents a highly efficient stimuli-responsive delivery system.

**Table 3.1.** The calculated MIC values of the lipid against both gram-positive and gram-negative bacteria.

| Bacterial strain           | MIC ( $\mu\text{M}$ ) |                  |                  |
|----------------------------|-----------------------|------------------|------------------|
|                            | SL1a                  | Dox@SL1a         | Only Dox         |
| <i>E. coli</i> ; MTCC-1687 | 32.00 $\pm$ 1.00      | 18.75 $\pm$ 4.00 | >72.00           |
| <i>S. aureus</i> ; MTCC-96 | 14.00 $\pm$ 1.00      | 10.00 $\pm$ 3.00 | 36.00 $\pm$ 5.00 |



**Figure 3.7.** The 96 well plate bacterial assay of Dox@3.SL1a (first and third row) against *E. coli* (MTCC-1687S). The clear wells specify inhibition of bacterial growth, while the cloudy wells indicate unconstrained bacterial growth. Field emission scanning electron microscope image of *E. coli* (MTCC-1687S) treated with Dox@3.SL1a (B) and only control bacterial cell (C).

### 3.3. Summary

Sulfonium-based modular lipids were successfully synthesized under mild reaction conditions. The potent cationic lipid self-assembled in an aqueous medium to form soluble spherical aggregates, which showed high phase transition

temperature, GSH-responsive cleavage, and other favorable biophysical properties of drug delivery agents. The sulfonium lipid displayed successful encapsulation and delivery of the hydrophobic anticancer drug Dox to MDA-MB-231 cells. Interestingly, the sulfonium lipid itself exhibits antimicrobial activities against both Gram-positive and Gram-negative bacteria. Hence, the sulfonium lipid could be a potential alternative to natural phospholipids, which can fight against bacterial infections and can be cleaved in a controlled fashion under the reductive tumor environment to release the encapsulated drug molecules.

### 3.4. Experimental section

#### 3.4.1. Synthesis of the compounds

##### 3.4.1.1. General information

All reagents were purchased from Sigma-Aldrich, Merck, Himedia, and other commercial sources and used directly without further purification. The column chromatography was performed using 60–120 mesh silica gels. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 (0.25 mm). The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR were recorded at 400 or 600 and 100 or 151 MHz with Varian AS400 spectrometer and Bruker spectrometer, respectively. The chemical shifts were reported in parts per million ( $\delta$ ) using  $\text{DMSO}-d_6$ ,  $\text{CDCl}_3$  as internal solvents. The coupling constant ( $J$  values) and chemical shifts ( $\delta_{\text{ppm}}$ ) were reported in Hertz (Hz) and parts per million (ppm), respectively, downfield from tetramethylsilane using residual chloroform ( $d = 7.24$  for  $^1\text{H}$  NMR,  $d = 77.23$  for  $^{13}\text{C}$  NMR) as an internal standard. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broadened). High-resolution mass spectra (HRMS) were recorded at Agilent Q-TOF mass spectrometer with a Z-spray source using built-in software for analysis of the recorded data. The 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL). Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers.

##### 3.4.1.2. General procedure for the synthesis of targeted compounds

###### 3.4.1.2.1. Synthesis of 1-(azidomethyl)-3,5-bis(bromomethyl)benzene (2)



To the stirring solution of 1,3,5- tris(bromomethyl)benzene (**3.1**) (1.40 mmol) in dry DMF solvent was slowly added, sodium azide (1.2 mmol), and the resulting mixture was stirred for 3 hours at room temperature. The progress of the reaction was monitored by TLC. After maximum consumption of compound **3.1**, the solvent was evaporated under reduced pressure, and CH<sub>2</sub>Cl<sub>2</sub> (25 mL) was added. The salts were filtered off, and the filtrate was concentrated under reduced pressure. The crude product was purified through silica gel column chromatography with a solvent gradient system using ethyl acetate and hexane (0-15% EtOAc in hexanes) to obtain the pure product as a clear yellow oil (200 mg; 45% yield). The compound was characterized by <sup>1</sup>H and <sup>13</sup>C NMR, which is in accordance with the literature report.<sup>38</sup>

#### 3.4.1.2.2. Synthesis of 1-(3,5-bis(bromomethyl)phenyl)-*N,N,N*-trimethylmethanaminium (**3.3**)

To the stirring solution of 1,3,5-tris(bromomethyl)benzene (1, 1.40 mmol) in acetone solvent was added trimethylamine (in 25% methanol solution; 1.2 mmol), and the resulting mixture was stirred for 12 hours at room temperature. A white precipitate was observed, which was filtered, and washed with acetone to afford the desired product as a white solid (380 mg, 80%). The compound was characterized by <sup>1</sup>H and <sup>13</sup>C NMR, which is in accordance with the literature report.<sup>39</sup>

#### 3.4.1.2.3. Synthesis of ((5-(azidomethyl)-1,3-phenylene)bis(methylene))bis(dodecylsulfane) (**3.4a**)

To a stirring solution of 1-(azidomethyl)-3,5-bis(bromomethyl)benzene (**3.2**, 0.31 mmol) in CH<sub>3</sub>CN/H<sub>2</sub>O (3:1 in volume) was slowly added (dropwise) a previously stirring solution of dodecanthiol and sodium bicarbonate in CH<sub>3</sub>CN/H<sub>2</sub>O (3:1 in volume) at room temperature. The resulting reaction mixture was stirred for 36 hours; after that, the solvent was removed under reduced pressure. Then the reaction mixture was diluted with cold water and ethyl acetate. The organic layer was extracted with EtOAc (3 × 50 mL) and washed with brine, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The organic solvent was removed under reduced pressure. The crude reaction mixture was purified through silica gel column chromatography with a solvent gradient system using ethyl acetate and hexane (0-10% EtOAc in hexanes) to afford compound **3.4a** as a colorless gummy liquid. Characterization of the

compound: Colourless gummy liquid (yield – 87%)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  7.23 – 7.22 (m, 1H), 7.14 (s, 1H), 4.31 (s, 2H), 3.69 – 3.66 (m, 4H), 2.42 – 2.37 (m, 4H), 1.57 – 1.50 (m, 4H), 1.35 – 1.24 (m, 36H), 0.88 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  139.7, 135.8, 129.3, 127.2, 54.5, 36.0, 31.9, 31.4, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.2, 28.9, 22.7, 14.1; HRMS (ESI) calcd. for  $\text{C}_{33}\text{H}_{63}\text{N}_4\text{S}_2$   $[\text{M}+\text{NH}_4]^+$  : 579.4494, found: 579.4463.

#### 3.4.1.2.4. Synthesis of ((5-(azidomethyl)-1,3-phenylene)bis(methylene))bis(hexadecylsulfane) (3.4b)

To a stirring solution of 1-(azidomethyl)-3,5-bis(bromomethyl)benzene (**3.2**, 0.31 mmol) in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (3:1 in volume) was slowly added (dropwise) a previously stirring solution of hexadecanthiol and sodium bicarbonate in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (3:1 in volume) at room temperature. The resulting reaction mixture was stirred for 36 hours; after that, the solvent was removed under reduced pressure. Then the reaction mixture was diluted with cold water and ethyl acetate. The organic layer was extracted with EtOAc (3  $\times$  50 mL) and washed with brine, and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The organic solvent was removed under reduced pressure. The crude reaction mixture was purified through silica gel column chromatography with a solvent gradient system using ethyl acetate and hexane (0-10% EtOAc in hexanes) to afford compound **3.4b** as a colorless gummy liquid. Characterization of the compound: colourless gummy liquid (yield – 83%)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  7.31 (m, 1H), 7.23 (m, 2H), 4.60 (s, 2H), 4.35 (s, 2H), 3.73 – 3.70 (m, 4H), 2.42 – 2.39 (m, 4H), 1.58 – 1.52 (m, 4H), 1.34 – 1.31 (m, 4H), 1.28 – 1.25 (m, 48H), 0.88 (t, 6H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  140.2, 138.4, 136.3, 128.8, 128.5, 126.7, 54.4, 45.7, 36.0, 35.9, 31.9, 31.6, 31.5, 29.7, 29.6, 29.6, 29.5, 29.5, 29.3, 29.2, 28.9, 28.8, 22.6, 14.1; HRMS (ESI) calcd. for  $\text{C}_{41}\text{H}_{79}\text{N}_4\text{S}_2$   $[\text{M}+\text{NH}_4]^+$  : 691.5746, found: 691.5738.

#### 3.4.1.2.5. Synthesis of 1-(3,5-bis((dodecylthio)methyl)phenyl)-*N,N,N*-trimethylmethanaminium (3.5a)

To a stirring solution of 1-(3,5-bis(bromomethyl)phenyl)-*N,N,N*-trimethylmethanaminium (**3.3**, 0.14 mmol) in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (3:1 in volume) was slowly added (dropwise) a previously stirring solution of dodecanthiol (0.30 mmol) and sodium bicarbonate (0.30 mmol) in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (3:1 in volume) at room

temperature. The resulting reaction mixture was stirred for 36 hours; after that, the solvent was removed under reduced pressure. Then the reaction mixture was diluted with cold water and ethyl acetate. The organic layer was extracted with EtOAc (3 × 50 mL) and washed with brine, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The organic solvent was removed under reduced pressure. The crude reaction mixture was purified through silica gel column chromatography with a solvent gradient system using ethyl acetate and hexane (0.1-10% EtOAc in hexanes) to afford compound **3.5a** as a colorless gummy liquid. Characterization of compound: colorless gummy liquid (yield – 81%) <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 7.41 (s, 3H), 4.92 (s, 2H), 3.70 (s, 4H), 3.41 (s, 9H), 2.42 (t, 4H), 1.58 – 1.50 (m, 4H), 1.35 – 1.24 (m, 39H), 0.87 (t, 6H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 140.5, 131.7, 127.7, 69.1, 52.9, 35.9, 32.1, 31.9, 29.7, 29.6, 29.6, 29.5, 29.3, 29.2, 29.2, 28.9, 22.6, 14.0; HRMS (ESI) calcd. for C<sub>44</sub>H<sub>84</sub>NS<sub>2</sub> [M]<sup>+</sup>: 578.4788, found: 578.4768.

#### 3.4.1.2.6. Synthesis of 1-(3,5-bis((hexadecylthio)methyl)phenyl)-*N,N,N*-trimethylmethanaminium (**3.5b**)

To a stirring solution of 1-(3,5-bis(bromomethyl)phenyl)-*N,N,N*-trimethylmethanaminium (**3.3**, 0.14 mmol) in CH<sub>3</sub>CN/H<sub>2</sub>O (3:1 in volume) was added dropwise a previously stirring solution of hexadecanethiol (0.30 mmol) and sodium bicarbonate (0.30 mmol) in CH<sub>3</sub>CN/H<sub>2</sub>O (3:1 in volume) at room temperature. The resulting reaction mixture was stirred for 36 hours; after that the solvent was removed under reduced pressure. Then the reaction mixture was diluted with cold water and ethyl acetate. The organic layer was extracted with EtOAc (3 × 50 mL) and washed with brine, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The organic solvent was removed under reduced pressure. The crude reaction mixture was purified through silica gel column chromatography with a solvent gradient system using ethyl acetate and hexane (0.1-10% EtOAc in hexanes) to afford compound **3.5b** as a colorless gummy liquid. Characterization of compound: colourless gummy liquid (yield – 81%) <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 7.43 – 7.41 (m, 3H), 4.88 (m, 2H), 3.73 – 3.71 (s, 4H), 3.39 – 3.37 (m, 9H), 2.45 – 2.41 (m, 4H), 1.57 – 1.52 (m, 4H), 1.34 – 1.24 (m, 54H), 0.87 (t, 3H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 140.7, 131.9, 127.8, 69.3, 53.1, 36.1, 32.2, 32.1, 29.9, 29.8, 29.7, 29.5, 29.3, 29.1, 22.9, 14.3; HRMS (ESI) calcd. for C<sub>44</sub>H<sub>84</sub>NS<sub>2</sub> [M]<sup>+</sup>: 690.6040, found: 690.6029.

#### 3.4.1.2.7. Synthesis of 3.SL1a

To a stirring solution of ((5-(azidomethyl)-1,3-phenylene)bis(methylene))bis(dodecylsulfane) (**3.4a**, 0.09 mmol) in dry DCM solvent was added  $\text{AgBF}_4$  (0.09 mmol) and methyl iodide (0.27 mmol) and the resulting mixture was stirred at room temperature for 5 hours. The progress of the reaction was monitored by TLC. After maximum consumption of compound **3.4a**, the solvent was removed under reduced pressure and the resulting mixture was filtered off using a pad of celite to remove silver salt. Then the crude mixture was washed with ether and  $\text{CH}_3\text{CN}$  to afford compound **3.SL1a** as a brown gummy liquid. Characterization of the compound: Brown gummy liquid (yield – 80%)  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta_{\text{ppm}}$  7.63 (s, 1H), 7.54 – 7.29 (m, 2H), 5.48 – 5.36 (m, 2H), 4.90 – 4.60 (m, 4H), 3.28 – 3.26 (m, 6H), 2.82 – 2.80 (m, 4H), 1.72 – 1.59 (m, 4H), 1.24 – 1.15 (m, 38H), 0.85 (t, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO}-d_6$ )  $\delta_{\text{ppm}}$  141.0, 133.0, 130.2, 101.9, 53.1, 44.5, 40.9, 31.7, 29.4, 29.4, 29.2, 29.1, 29.1, 28.7, 28.2, 23.7, 22.5, 14.4; ES-MS (ESI+)  $m/z$ :  $[(M + \text{NH}_4^+)/2]$  305.2431.

#### 3.4.1.2.8. Synthesis of 3.SL1b

To a stirring solution of ((5-(azidomethyl)-1,3-phenylene)bis(methylene))bis(hexadecylsulfane) (**3.4b**, 0.09 mmol) in dry DCM solvent was added  $\text{AgBF}_4$  (0.09 mmol) and methyl iodide (0.27 mmol) and the resulting mixture was stirred at room temperature for 5 hours. The progress of the reaction was monitored by TLC. After maximum consumption of compound **3.4b**, the solvent was removed under reduced pressure and the resulting mixture was filtered off using a pad of celite to remove silver salt. Then the crude mixture was washed with ether and  $\text{CH}_3\text{CN}$  to afford compound **3.SL1b** as a brown gummy liquid. Characterization of the compound: Brown gummy liquid (yield – 72%)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  8.09 – 7.77 (m, 2H), 7.63 – 7.41 (m, 1H), 4.75 – 4.64 (m, 4H), 4.57 – 4.40 (m, 2H), 3.43 – 3.19 (m, 6H), 2.87 – 2.73 (m, 4H), 1.84 – 1.63 (m, 4H), 1.37 – 1.20 (m, 51H), 0.86 (t, 6H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  130.4, 130.2, 101.9, 53.1, 44.5, 40.9, 31.7, 29.5, 29.4, 29.4, 29.2, 29.1, 28.7, 28.2, 23.7, 22.5, 14.4; ES-MS (ESI+)  $m/z$ :  $[(M + 2\text{BF}_4^-)/2]$  439.2758.

#### 3.4.1.2.9. Synthesis of 3.SL2a

To stirring solution of 1-(3,5-bis((dodecylthio)methyl)phenyl)-N,N,N-trimethylmethanaminium (**3.5a**, 0.05 mmol) in dry DCM solvent was added AgBF<sub>4</sub> (0.05 mmol) and methyl iodide (0.15 mmol) and the resulting mixture was stirred for 5 hours at room temperature. The progress of the reaction was monitored by TLC. After maximum consumption of maximum amount of compound **3.5a**, the solvent was removed under reduced pressure and the resulting mixture was filtered off using a pad of celite to remove silver salt. Then the crude mixture was washed with ether and CH<sub>3</sub>CN to afford compound **3.SL2a** as a brown gummy liquid. Characterization of compound: Brown gummy liquid (yield – 77%); <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ<sub>ppm</sub> 7.75- 7.56 (m, 3H), 4.86 – 4.73 (m, 4H), 4.60 – 4.54 (m, 2H), 3.07 – 3.04 (m, 9H), 2.85 – 2.80 (m, 6H), 1.74 – 1.67 (m, 4H), 1.36 - 1.24 (m, 40H), 0.85 (t, 3H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>) δ<sub>ppm</sub> 136.4, 130.6, 129.7, 89.9, 70.1, 68.3, 52.6, 47.4, 44.6, 41.4, 32.1, 30.2, 30.0, 29.9, 29.7, 29.6, 28.6, 24.2, 22.9, 21.1, 14.3; ES-MS (ESI+) *m/z*: [(M + H)<sup>+</sup> /2] 305.2433.

#### 3.4.1.2.10. Synthesis of 3.SL2b

To stirring solution of 1-(3,5-bis((hexadecylthio)methyl)phenyl)-N,N,N-trimethylmethanaminium (**3.5b**, 0.05 mmol) in dry DCM solvent was added AgBF<sub>4</sub> (0.05 mmol) and methyl iodide (0.15 mmol) and the resulting mixture was stirred for 5 hours at room temperature. The progress of the reaction was monitored by TLC. After maximum consumption of compound **3.5b**, the solvent was removed under reduced pressure and the resulting mixture was filtered off using a pad of celite to remove silver salt. Then the crude mixture was washed with ether and CH<sub>3</sub>CN to afford compound **3.SL2b** as a brown gummy liquid. Characterization of compound: brown gummy liquid (yield – 76%) <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 7.73 – 7.49 (m, 3H), 4.60 – 4.44 (m, 4H), 3.46 – 2.95 (m, 9H), 2.81 – 2.73 (m, 6H), 1.85 - 1.71 (m, 5H), 1.49 – 1.40 (m, 2H), 1.31 – 1.17 (m, 52H), 0.88 (t, 3H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>) δ<sub>ppm</sub> 136.4, 130.6, 129.7, 89.9, 70.1, 68.3, 52.6, 47.4, 44.6, 41.4, 32.1, 30.2, 30.0, 29.9, 29.7, 29.6, 28.6, 24.2, 22.9, 21.1, 14.3; ES-MS (ESI+) *m/z*: [(M + BF<sub>4</sub> - ) + /2] 404.3398.

#### 3.4.1.2.11. Synthesis of 3.SL3a



To a stirring solution of ((5-(azidomethyl)-1,3-phenylene)bis(methylene))bis(dodecylsulfane) (**3.4a**, 0.09 mmol) in DMF (3 mL) was added prop2-yne-1-sulfonic acid (0.13 mmol) and the mixture was stirred for 10 minutes. After that, sodium ascorbate (0.003 mmol) and CuI (0.002 mmol) were added to the reaction mixture, and the solution was allowed to stir for 24 hours at room temperature. After maximum consumption of compound **3.4a**, the unused solvent was removed under reduced pressure and diluted with ethyl acetate. The organic layer was separated, washed with brine, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solid crude product was washed with distilled hexane. The crude reaction mixture was purified through silica gel column chromatography with a solvent gradient system using methanol and DCM (0.1-10% methanol in DCM) to afford compound (1-(3,5-bis((dodecylthio)methyl)benzyl)-1H-1,2,3-triazol-4-yl)methanesulfonic acid (**3.6a**) as a white solid which was used for next reaction without further characterization. To a stirring solution of compound **6a** in dry DCM was added AgBF<sub>4</sub> (0.06 mmol), and methyl iodide (0.18 mmol), and the resulting mixture was stirred for 5 hours at room temperature. The progress of the reaction was monitored by TLC. After maximum consumption of compound **3.6a**, the solvent was removed under reduced pressure, and the resulting mixture was filtered off using a pad of celite to remove silver salt. Then the crude mixture was washed with ether and CH<sub>3</sub>CN to afford compound **3.SL3a** as a brown gummy liquid. Characterization of compound: Dark Brown gummy liquid (yield – 71%) <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>)  $\delta_{\text{ppm}}$  9.78 (s, 1H), 8.00 – 7.93 (m, 3H), 4.69 – 4.67 (m, 2H), 3.75 – 3.71 (m, 2H), 3.33 – 3.20 (m, 4H), 2.78 (s, 6H), 2.35 – 2.28 (m, 1H), 1.85 – 1.71 (m, 4H), 1.44 – 1.40 (m, 4H), 1.29 – 1.23 (m, 41H), 0.86 (t, 6H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>)  $\delta_{\text{ppm}}$  138.5, 133.2, 130.3, 129.2, 128.4, 125.5, 68.1, 47.4, 44.8, 41.6, 32.1, 29.9, 29.9, 29.8, 29.6, 29.2, 28.6, 25.8, 24.3, 22.9, 21.5, 14.3; ES-MS (ESI+) m/z: [(M/2 + 2BF<sub>4</sub><sup>-</sup> + Na<sup>+</sup>) ] 552.0522.

#### 3.4.1.2.12. Synthesis of SL3b

To a stirring solution of ((5-(azidomethyl)-1,3-phenylene)bis(methylene))bis(hexadecylsulfane) (**3.4b**, 0.07 mmol) in DMF (3 mL) was added prop-2-yne-1-sulfonic acid (0.11 mmol) and the mixture was stirred for 10 minutes. After that, sodium ascorbate (0.003 mmol) and CuI (0.002 mmol) were

added to the reaction mixture, and the solution was allowed to stir for 24 hours at room temperature. After maximum consumption of compound **3.4b**, the unused solvent was removed under reduced pressure and diluted with ethyl acetate. The organic layer was separated, washed with brine, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solid crude product was washed with distilled hexane. The crude reaction mixture was purified through silica gel column chromatography with a solvent gradient system using methanol and DCM (0.1-10% methanol in DCM) to afford compound (1-(3,5-bis((hexadecylthio)methyl)benzyl)-1H-1,2,3-triazol-4-yl)methanesulfonic acid (**3.6b**) as a white solid which was used for next reaction without further characterization. To a stirring solution of compound **3.6b** in dry DCM was added AgBF<sub>4</sub> (0.06 mmol), and methyl iodide (0.18 mmol), and the resulting mixture was stirred for 5 hours at room temperature. The progress of the reaction was monitored by TLC. After maximum consumption of compound **6b**, the solvent was removed under reduced pressure, and the resulting mixture was filtered off using a pad of celite to remove silver salt. Then the crude mixture was washed with ether and CH<sub>3</sub>CN to afford compound **3.SL3b** as a brown gummy liquid. Characterization of compound: Dark brown gummy solid (yield – 68%) <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 9.80 – 9.76 (m, 1H), 8.00 – 7.99 (m, 2H), 7.96 (s, 1H), 4.73 – 4.63 (m, 2H), 3.70 (s, 4H), 2.92 – 2.67 (m, 6H), 2.52 – 2.51 (m, 2H), 1.79 – 1.72 (m, 4H), 1.64 – 1.57 (m, 4H), 1.45 – 1.40 (m, 4H), 1.31 – 1.25 (m, 56H), 0.87 (t, 3H); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ<sub>ppm</sub> 138.6, 138.3, 133.1, 130.4, 67.2, 58.9, 47.2, 40.2, 34.2, 32.1, 29.9, 29.8, 29.7, 29.5, 29.2, 28.5, 24.8, 24.3, 22.8, 21.6, 19.8, 14.3; ES-MS (ESI+) m/z: [(M/3 + Na<sup>+</sup>) 298.2419.

### 3.4.2. Preparation of the lipid vesicles

Vesicles were prepared by thin film hydration method in 20 mM HEPES buffer, pH 7.4, containing 100 mM KCl.<sup>40</sup> First, 40-50 µL solution of the lipid was taken from 10 mM lipid stock and dried under reduced pressure for 3 hours to prepare the lipid film. Then 500 µL of the HEPES buffer pH 7.4 was added into the lipid film and heated to 60-70 °C for 10 min (final concentration of the liposome ~1 mM). After that, the solution was vortexed well until the dry lipid film mostly disappeared. Finally, the solution was sonicated 10 times (30 s of sonication followed by 30 s of cooling on ice). A hand-held mini extruder (Avanti Polar Lipids, Alabaster, AL) with



polycarbonate membrane (diameter of 200 nm) was used to prepare small unilamellar vesicles (at room temperature) whenever required. We observed that hydration of the dried film of the **3.SL1a** lipids over 60-70 °C only resulted in the formation of soluble aggregates.

### 3.4.3. Transmission electron microscopy analysis

The morphology of the aqueous soluble aggregates of the lipids was investigated by transmission electron microscope (TEM). For TEM imaging, the lipid solution was prepared by the above-mentioned method in HEPES buffer, pH 7.4.<sup>41-42</sup> The prepared lipid solution (without extrusion) was diluted to half of its original concentration using HEPES buffer, pH 7.4. From that diluted solution, only 10  $\mu$ L of lipid solution was placed onto a carbon-coated copper grid and allowed to absorb for 1 minute. The grid was then carefully blotted with filter paper, and only a trace amount of the solution in the middle of the grid was kept. After that, the grid was allowed to dry for 10 minutes at 30 °C. Finally, 5-10  $\mu$ L of 2% uranyl acetate solution (in water) was added on the grid and allowed to dry for another 1 min. The excess uranyl acetate solution was wicked off, and the grid was dried overnight at 30 °C. The images were collected using a JEOL JEM 2100 transmission electron microscope (operated at a maximum accelerating voltage of 200 kV).

### 3.4.4. Lipid phase transition temperature measurements

To determine the phase transition temperature ( $T_m$ ) from the ordered gel to the liquid disordered phase of the lipids, the temperature-dependent steady-state anisotropy measurements were performed.<sup>41-42</sup> The environment-sensitive dye 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) was used as the fluorescence probe to measure the change in anisotropy values. All the vesicles were prepared according to the method described in the previous section (the hydration was done by using 20 mM HEPES buffer, pH 7.4, containing 100 mM KCl). After that, the lipid solution (1000  $\mu$ L) and DPH solution (10  $\mu$ L of 1 mM stock solution in THF) were taken in a microcentrifuge tube (final concentration of DPH was 10  $\mu$ M; DPH < 1% v/v in vesicle solution). The solution was kept at room temperature under tumbling conditions for overnight to ensure the maximum incorporation of DPH molecules inside the hydrophobic core of the lipid bilayer. Then steady-state fluorescence anisotropy

measurements were performed with a refrigerated system using a Peltier temperature controller connected to a fluoromax-4 spectrofluorometric (Horiba Scientific). The degree ( $r$ ) of anisotropy in the DPH fluorescence ( $\lambda_{\text{ex}} = 350 \text{ nm}$ ;  $\lambda_{\text{em}} = 429 \text{ nm}$ ) was calculated using the following equation (eq. 1) at the peak of the fluorescence spectrum. The  $I_{\text{VV}}$  and  $I_{\text{VH}}$  are the fluorescence intensities of the emitted light polarized parallel and perpendicular to the excited light, respectively, and  $G = I_{\text{VH}}/I_{\text{HH}}$  is the instrumental grating factor.

$$r = \frac{(I_{\text{VV}} - G I_{\text{VH}})}{(I_{\text{VV}} + 2G I_{\text{VH}})} \quad (\text{eq. 1})$$

At every 3-4 °C temperature difference, the anisotropy values were recorded, apart from the near to the  $T_{\text{m}}$  value of the lipid; in that case, the anisotropy values were collected at every 1 or 2 °C. All anisotropy values of the DPH probe are the mean values of three individual measurements. Finally, to determine the  $T_{\text{m}}$  value of the lipid plots, the degree of anisotropy ( $r$ ) of the DPH probe as a function of temperature was investigated.

#### 3.4.5. pH-dependent particle size and surface-potential measurement of the liposome

The vesicles were prepared according to the method as described in the earlier section in 25 mM HEPES buffer, pH 7.4, containing 100 mM KCl.<sup>41-42</sup> Different buffer solutions were freshly prepared using isosmotic buffers consisting of 10 mM buffering agent and 10 mM salt to vary the pH range (pH 3 to pH 8.5) and used for the measurements. Citric acid and trisodium-citrate for pH 3.0–6.5; 3 (n-morpholino) propanesulfonic acid (MOPS) for pH 7.0, and tris(hydroxymethyl)aminomethane (Tris)-HCl were used for pH 7.5–8.5. Both the zeta potential and hydrodynamic diameter of the vesicles at different pH were measured using a Zetasizer Nano ZS90 (Malvern, Westborough, MA) instrument at 25 °C.

#### 3.4.6. Dealkylation of 3.SL1a lipid in the presence of glutathione

The lipid **3.SL1a** (1 mM) was dissolved in 25 mM HEPES buffer, pH 7.4 containing 10 mM reduced GSH, and the mixture was incubated in 37 °C. At different time intervals, the same amount of aliquot of the reaction solution was taken out, and HPLC analysis was performed. The aliquot was dissolved in methanol before its

injection to the analytical HPLC (Waters 600E HPLC) system. The Hypersil GOLD™ C18 Selectivity LC Column and a UV-detector (254 nm) were used for the analysis. Acetonitrile/water isocratic gradient (95/5 (v/v)) was used as the mobile phase at a flow rate of 1.0 mL/minute for 10 minutes run time. Fragmented product was collected, and the identity of the product was confirmed by ES-MS (ESI+) analysis.

### 3.4.7. Doxorubicin encapsulation and release efficiency measurement

In a glass vial, the **3.SL1a** lipid (50  $\mu$ L from a stock solution of 28 mM in chloroform) was taken and dried under N<sub>2</sub> atmosphere for 4 hours to form multilayers. Then, 10  $\mu$ L of Dox hydrochloride (stock solution of 2 mM in water) was added into the same vial and hydrated with 1 mL of 25 mM HEPES buffer, pH 7.4, containing 100 mM KCl. After that, the solution was vortexed well for 15-20 minutes and then sonicated, as mentioned earlier.<sup>41-42</sup> The unencapsulated free Dox was separated by centrifuge followed by three times washing with the same buffer HEPES buffer, pH 7.4. After that, the spectroscopic measurements were performed, and finally, the vesicles were lysed with 2 mM Triton X-100 to release the Dox molecules. From the UV-Vis spectra, the concentration of Dox in free, encapsulated (inside the vesicles), and released (from the vesicles) state was calculated. From the calibration curve, the extinction coefficient ( $\epsilon$ ) was found  $0.00781 \mu\text{M}^{-1}\text{cm}^{-1}$  for Dox.

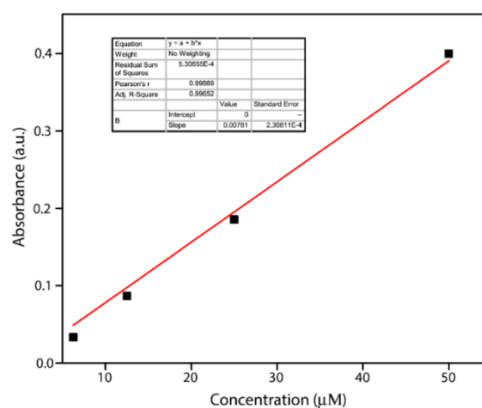
According to the Lambert-beer's law,  $A = \epsilon \cdot C_{f1} \cdot l$ .

Where  $C_{f1}$  is the concentration of Dox after complete release from the **3.SL1a** lipid vesicles at pH 7.4.

The initial concentration of Dox ( $C_i$ ) was 20  $\mu$ M. Path length ( $l$ ) = 1 cm.

So,  $C_{f1} = A/\epsilon \cdot l = 0.055094/0.00781 \times 1 = 7.054 \mu\text{M}$ .

Thus, Dox encapsulation efficiency at pH 7.4 =  $(C_{f1}/C_i) \times 100 = 35.3 \%$ .



**Figure 3.8.** Calculation of extinction coefficient ( $\epsilon$ ) for Dox.

To measure the Dox release profile, the vesicles were prepared by the same thin-film hydration method as previously mentioned in 25 mM HEPES buffer, pH 7.4, containing 100 mM KCl, in the presence of Dox. After that, the free Dox molecules were separated by centrifuge followed by three times washing with the same buffer. Then the loaded Dox loaded vesicle was diluted to 1 mM in the presence and absence of GSH (10 mM) containing the same buffer solution. The release of Dox molecules from the vesicles was measured by monitoring the emission signals at 516 nm ( $\lambda_{\text{ex}} = 485 \text{ nm}$ ) in the Fluoromax-4 spectrofluorometer at room temperature. Finally, the vesicles were lysed using Triton X-100 surfactant to measure the fluorescence of total encapsulated Dox.

### 3.4.8. Cell culture

The MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) supplemented with 10% Fetal Bovine Serum (FBS, GIBCO) and penicillin-streptomycin solution (GIBCO; 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin). Cells were maintained in a humidified atmosphere of 5%  $\text{CO}_2$  at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator. In all experiments, the medium was changed daily with complete media.

### 3.4.9. Determination of cell viability using MTT assay

The MDA-MB-231 cells were cultured, as mentioned earlier. For the cell viability study, cells were seeded in 96-well flat-bottomed plates at a density of  $4 \times 10^3$  cells per well and incubated for 24 hours in complete media in a  $\text{CO}_2$  incubator. Thereafter, cells were treated with different concentrations of **3.SL1a**, free Dox, and

Dox@**3.SL1a** for 48 or 72 hours (as per the experimental design mentioned in the figure legend) in 1% FBS containing media. The cell viability was then assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma-Aldrich) assays. Briefly, media was discarded, and cells were treated with MTT (final concentration: 0.5 mg/ml) for 2 hours at 37°C in a CO<sub>2</sub> incubator, and the resulting purple-colored formazan crystals formed were dissolved in 200 µL of DMSO. Cell viability was then measured using Multiscan GO (Thermo Scientific) at 540 nm. Cell viability (%) = (fluorescent intensity (sample)) / (fluorescence intensity (control)) × 100%.

#### 3.4.10. Determination of cellular uptake using microscopy analysis

The MDA-MB-231 cells were cultured, as mentioned earlier. For microscopy analysis, cells were seeded in 6-well plates at a density of  $1.5 \times 10^5$  cells per well. Each well contained a sterile 22 mm square cover slip (pre-coated with 0.1% gelatin) in the bottom and incubated overnight in complete media in a CO<sub>2</sub> incubator. Cells were then treated with Dox@**3.SL1a** at their respective IC<sub>50</sub> concentration for 8 hours in 1% FBS-containing media. Post-treatment, cells were washed with PBS buffer and fixed with 4% formaldehyde at 37 °C for 10 minutes. Cover slips were then washed thrice with PBS to wash out formaldehyde. Further, cells were treated with 0.1% Triton X-100 for 5 minutes at 37 °C. Cells were again washed thrice with PBS buffer to remove triton X-100. To counterstain nuclei, DAPI (5 µg/mL) was added and incubated for 5 minutes. Cells were then washed thrice with PBS. Subsequently, cover slips were taken out from the wells and mounted over a drop of FluorPreserve Reagent (Sigma) over a glass slide. The slides were then allowed to dry for 15 mins. Finally, microscopy was performed using confocal laser scanning microscope (Leica TCS SP8;  $\lambda_{\text{ex}} = 488 \text{ nm}$ ,  $\lambda_{\text{em}} = 560\text{--}630 \text{ nm}$  for Dox and  $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}} = 440\text{--}480 \text{ nm}$  for DAPI).

#### 3.4.11. Determination of cellular uptake using flow cytometry

The MDA-MB-231 cells were cultured, as mentioned earlier. Cells were seeded in 6-well plates at a density of  $1.5 \times 10^5$  cells per well and incubated for 24 hours in complete media. The cells were then treated with Dox@**3.SL1a**, and free Dox were treated with their respective IC<sub>50</sub> concentration in 1% FBS containing media for 4



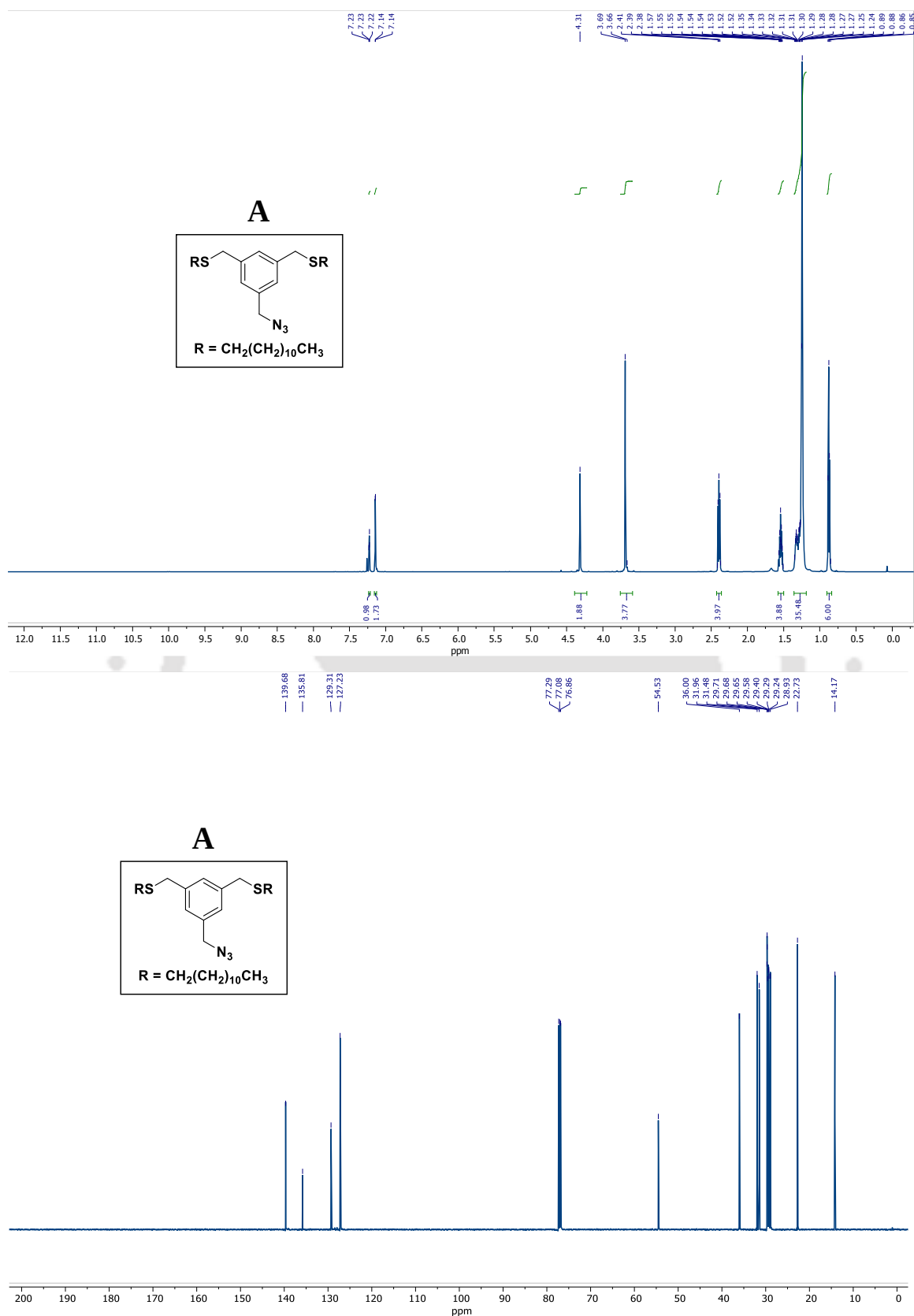
and 8 hours at 37°C. Next, the cells were washed twice with PBS (GIBCO), and trypsinised (0.05%; GIBCO) for 2 minutes at 37 °C followed by 5 min centrifugation at 800 rpm at 4°C. The supernatant was discarded, and cell pellets were re-suspended in 300uL of PBS (supplemented with 2% FBS). Finally, the solutions were kept on ice, and drug uptake by the MDA-MB-321 cells was analyzed by flow cytometry analysis using BD FACS Calibur Flow Cytometer (BD Biosciences). Data analysis was performed by the FCS Express 5 Flow Cytometry software.

#### 3.4.12. Measurement of antibacterial activity

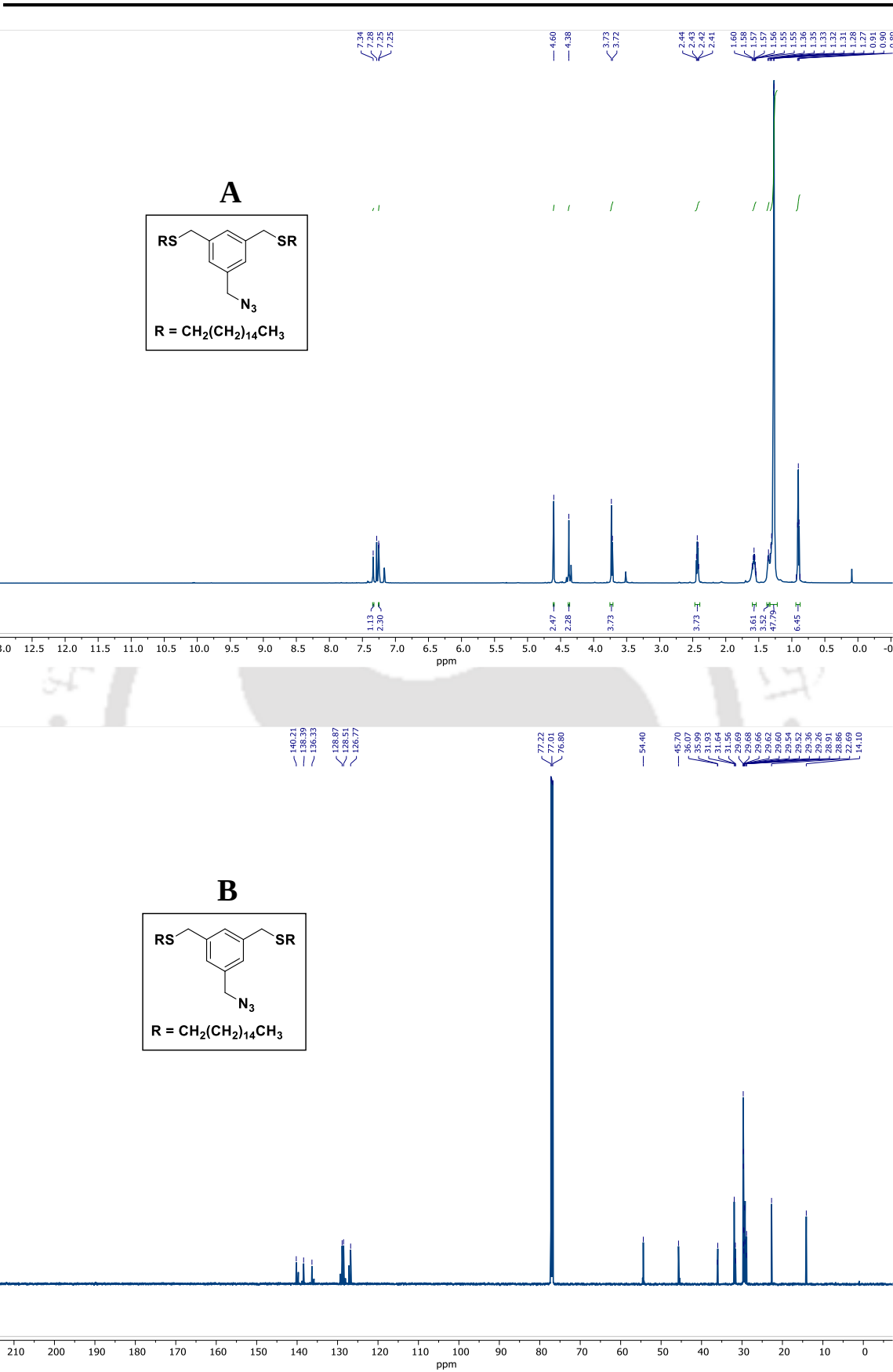
The minimum inhibitory concentration (MIC) of **3.SL1a** lipid was measured by micro broth dilution method against both gram-negative (*E. coli*; MTCC-1687) and gram-positive (*S. aureus*; MTCC-96) bacteria. The *E. coli* and *S. aureus* microbes were grown in Luria Bertani (LB), and Brain Heart Infusion (BHI) broth media, respectively at 37°C and 180 rpm in a shaker for overnight and a fresh media were inoculated from the overnight grown culture till the OD reached up to 0.5 to 0.6. The cells were then harvested by centrifugation and added to the media at a 10<sup>6</sup> CFU/mL cell concentration. Then, the cells were added to the wells containing serially diluted sulfonium lipid (**3.SL1a**) in 96 well plates. The plate was incubated for 14 to 16 h at 37 °C, and the absorbance of the plate was measured by using a Tecan infinite M200 Plate reader at 600 nm. The MIC values of the Dox and Dox@**3.SL1a** treated bacterial cells were also measured using a similar method.

#### 3.4.13. Morphological change analysis of the bacterial cells

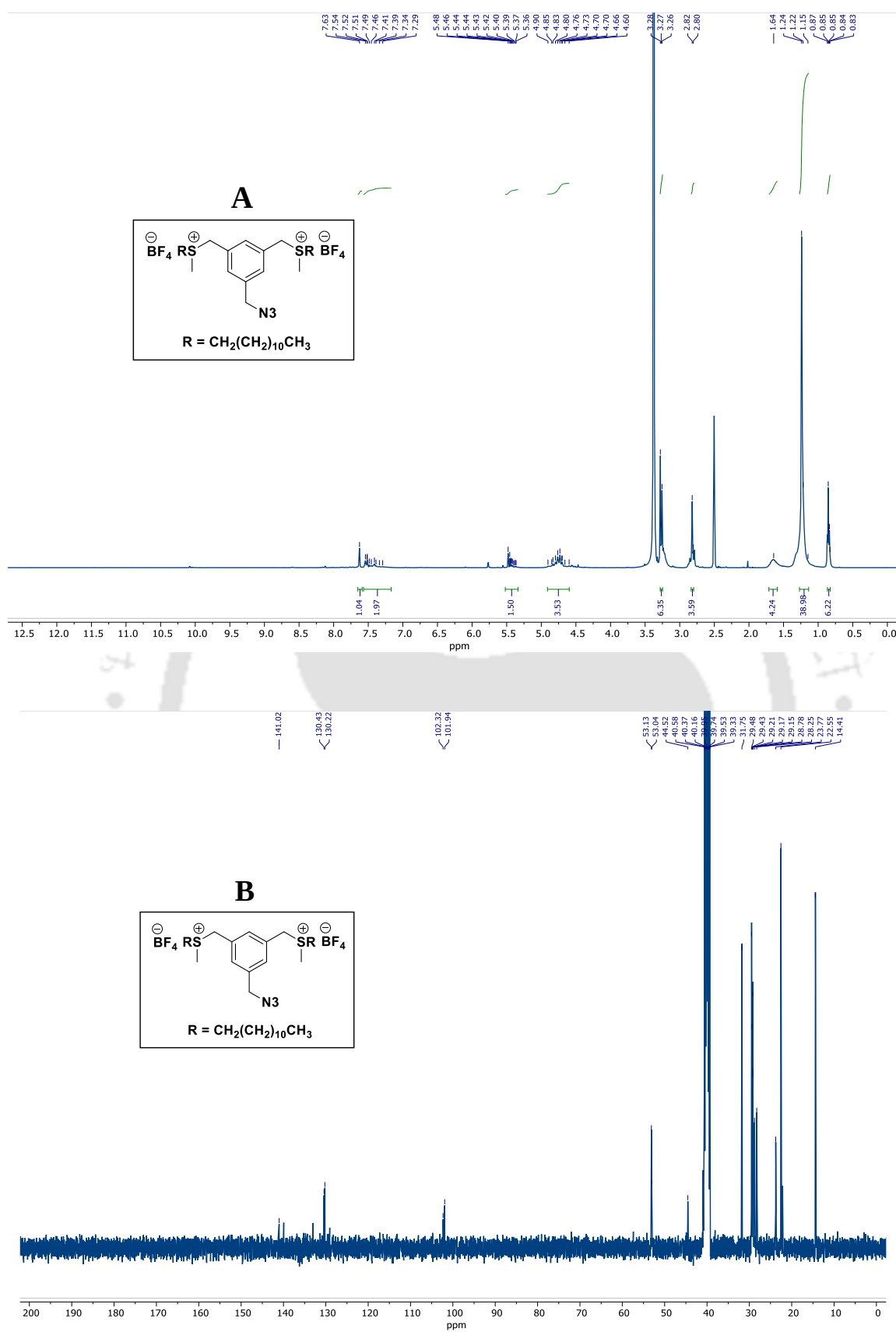
The morphology of the bacterial cells in the absence or the presence of the chemicals was investigated by FESEM analysis. The *E. coli* cells were grown in media, as mentioned in the above experimental section. The cells were treated by the lipid at a concentration of its MIC value and control and incubated for 5 h at 37°C. The bacterial cells were collected by centrifugation and washed with PBS pH 7.4 buffer followed by Mili-Q water, and then it was drop cast onto the aluminum foil wrapped on a glass grid.

3.4.14.  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of synthesized compoundsFigure 3.9.  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid 3.4a.





**Figure 3.10.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid **3.4b**.



**Figure 3.11.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid **3.SL1a**.

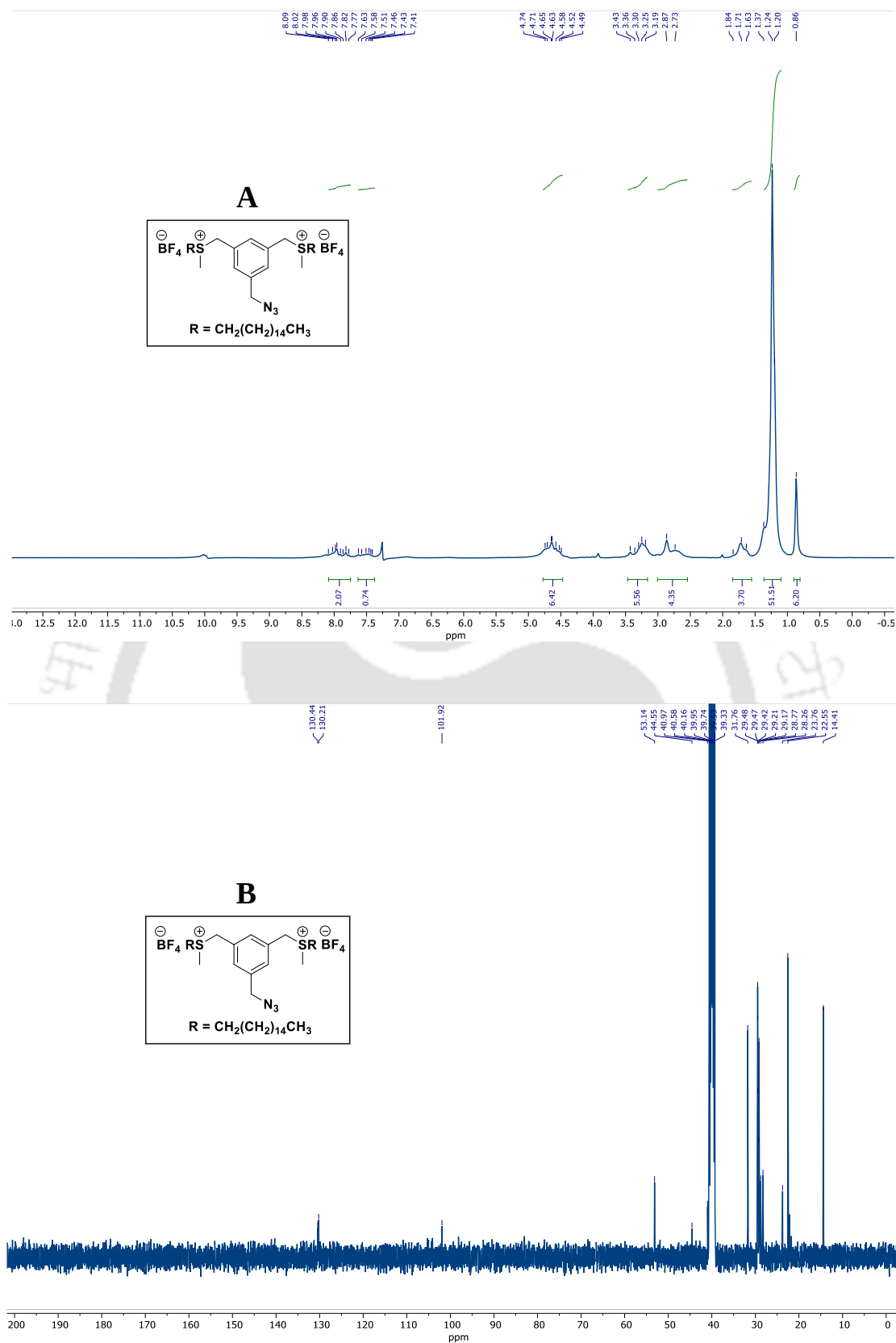
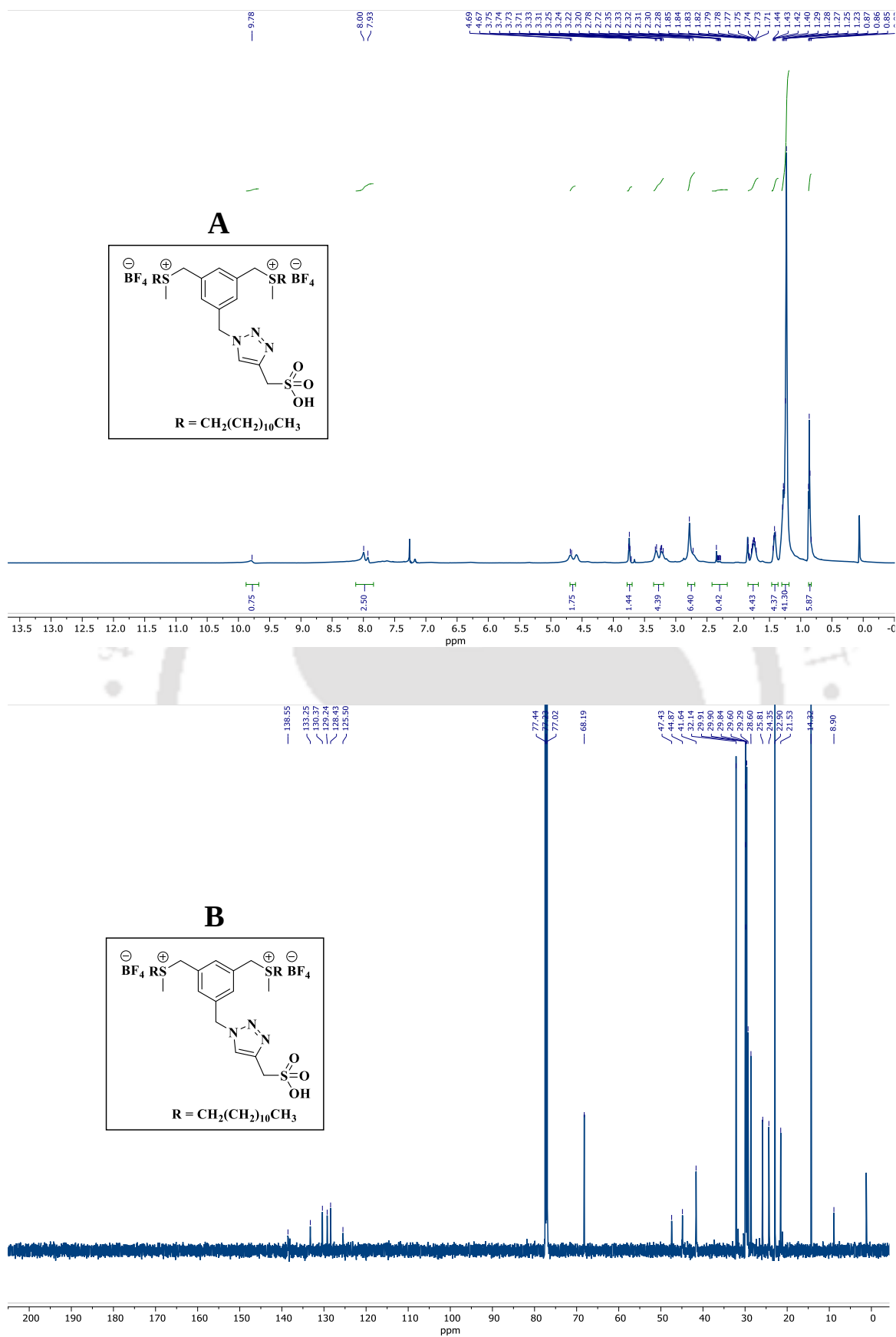
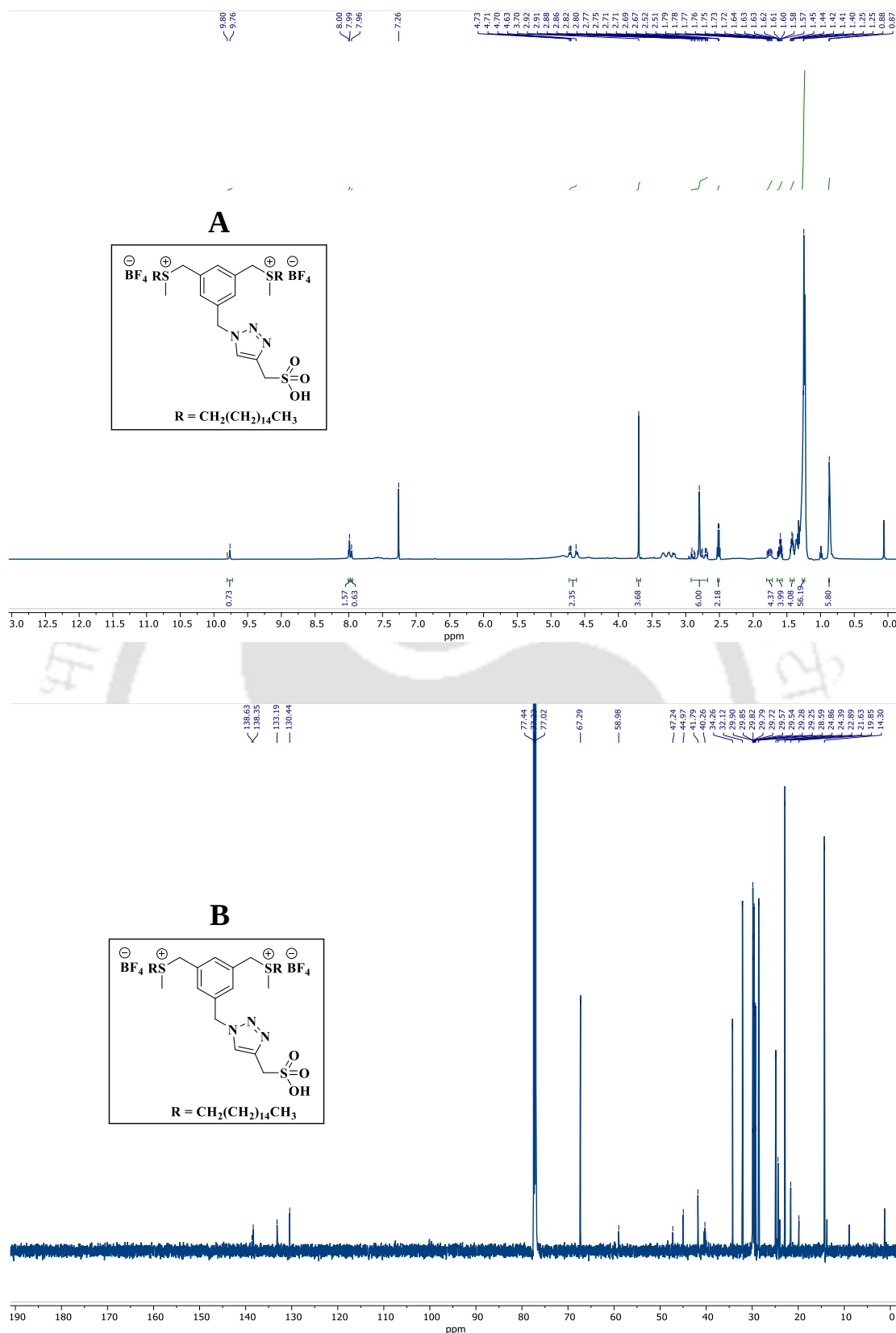


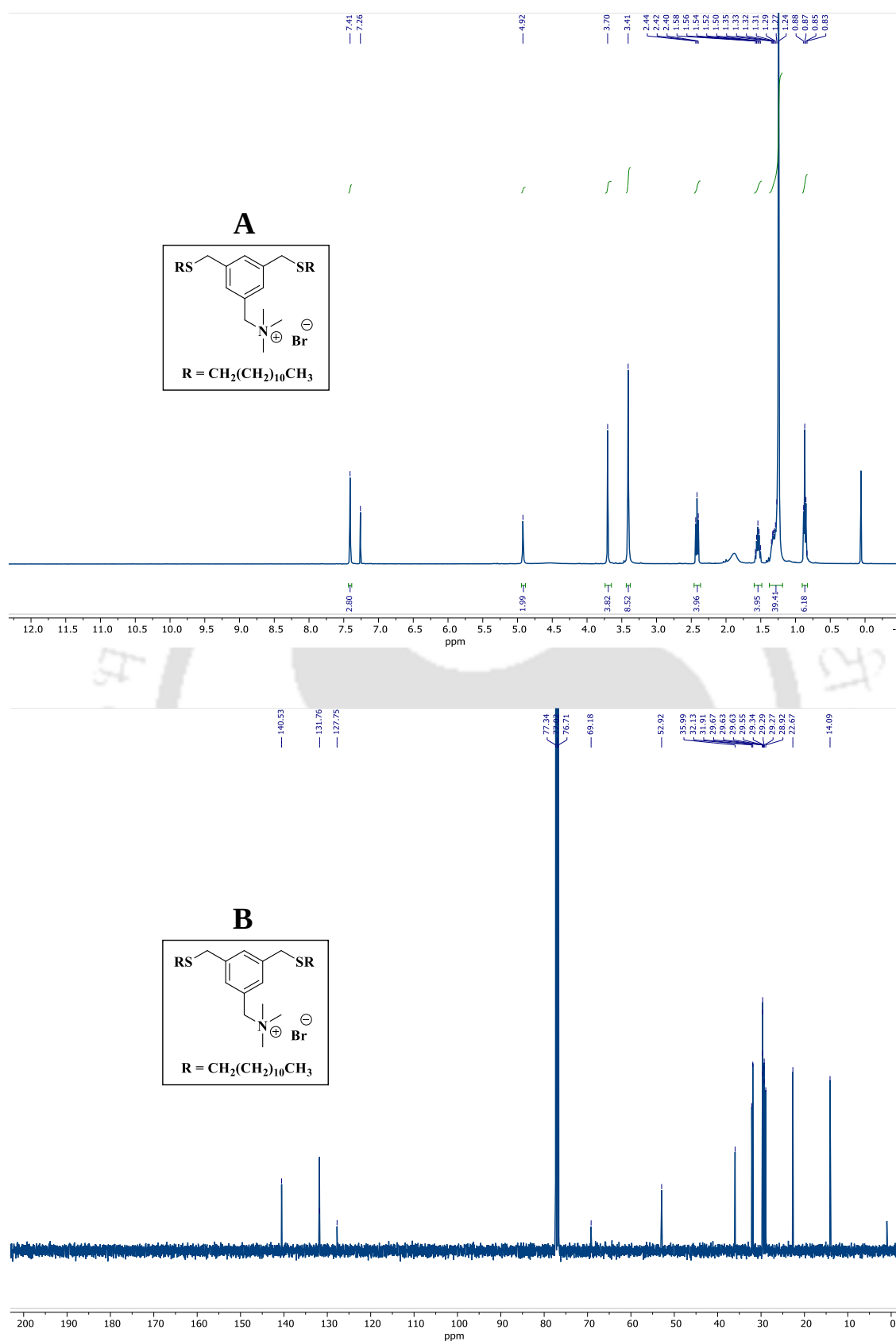
Figure 3.12. <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of lipid 3.SL1b.



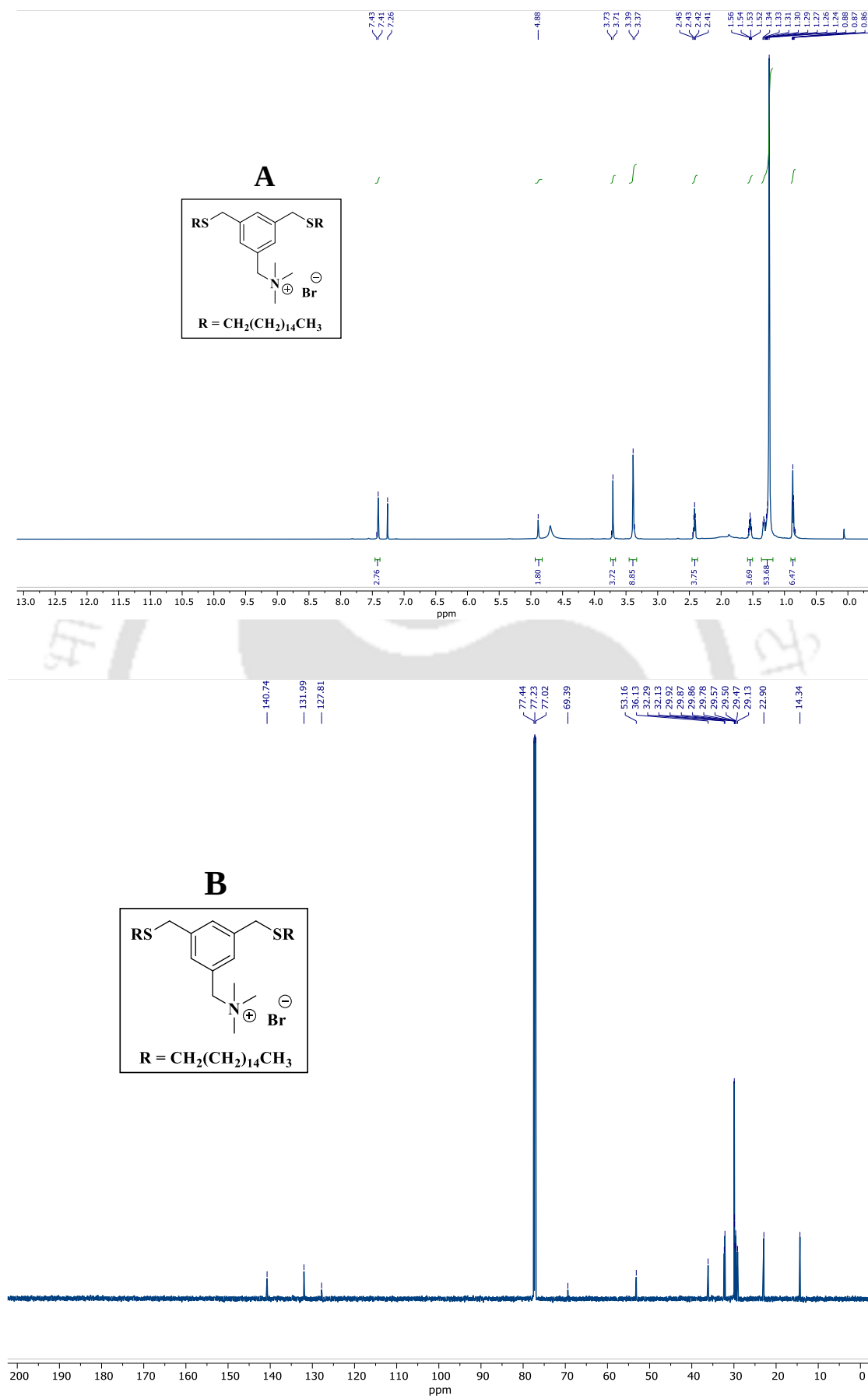
**Figure 3.13.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid 3.SL3a.



**Figure 3.14.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of lipid **3.SL3b**.



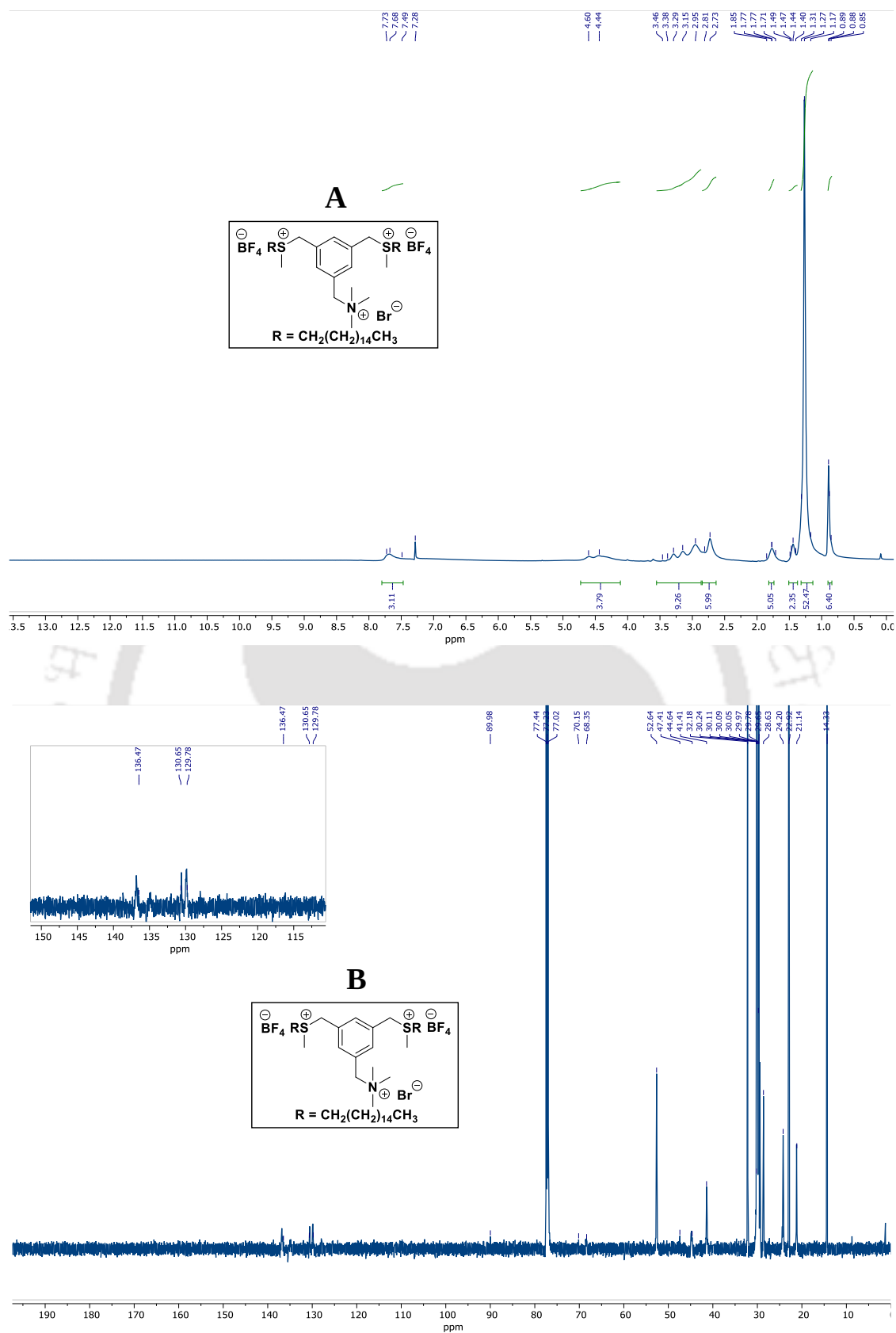
**Figure 3.15.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of **3.5a**



**Figure 3.16.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of **3.5b**

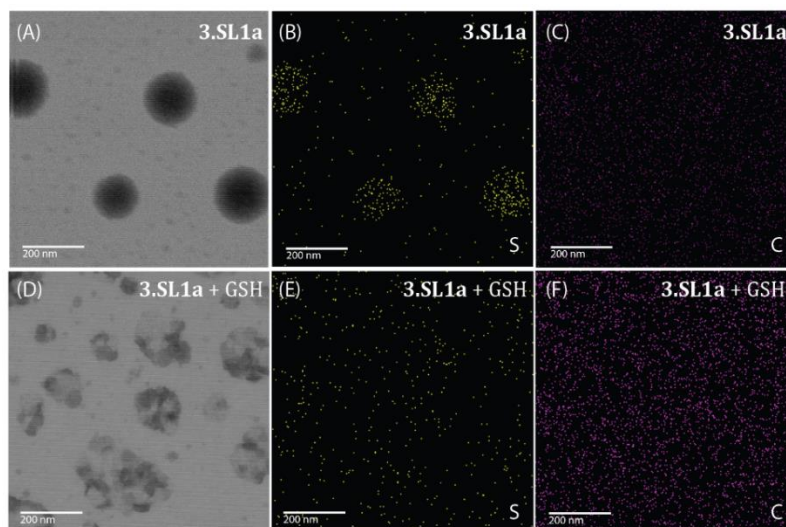






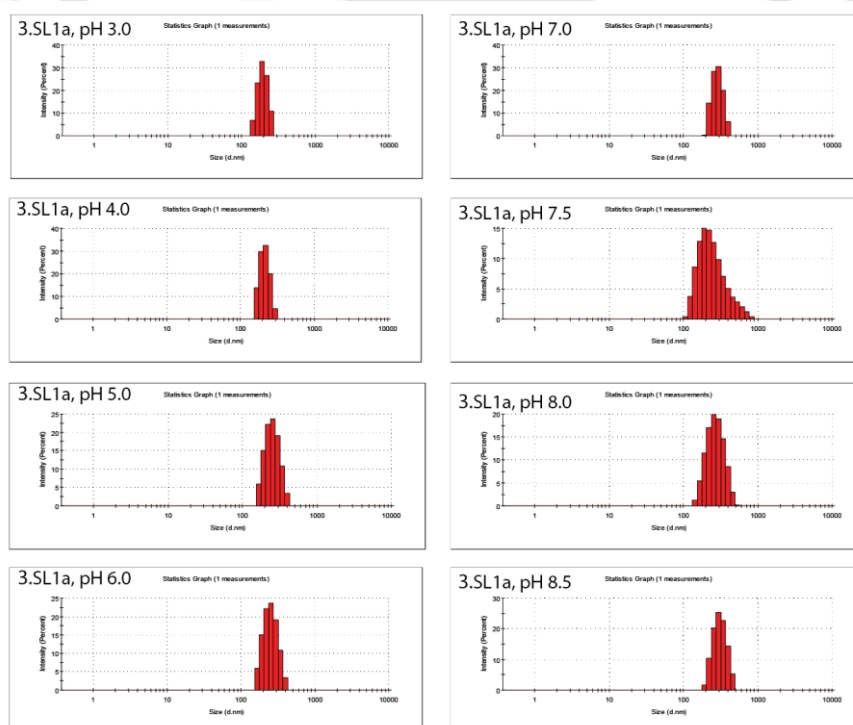
**Figure 3.18.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of **3.SL2b**.

### 3.4.15. TEM-EDX analysis of the lipid vesicle before and after glutathione treatment



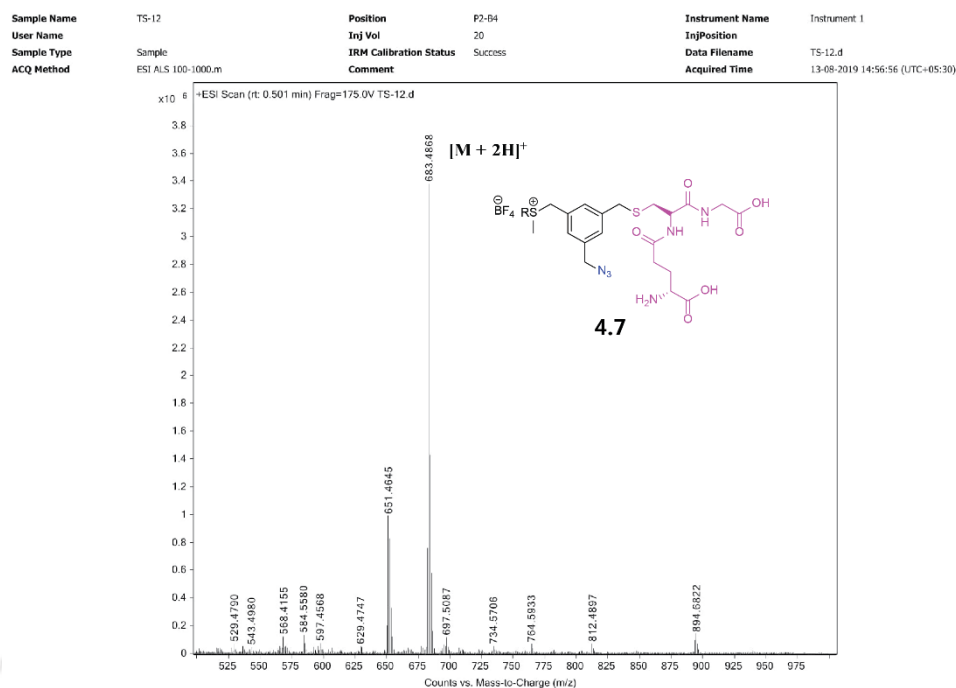
**Figure 3.19.** TEM-EDX bright field image of 100% **3.SL1a** lipid in absence and presence of 10 mM GSH (A and D). Carbon TEM-EDX elemental mapping of 100% **3.SL1a** lipid in absence and presence of GSH (B and E). Sulphur TEM-EDX elemental mapping of 100% **3.SL1a** lipid in absence and presence of GSH (C and F).

### 3.4.16. Size distribution analysis of the lipid vesicle

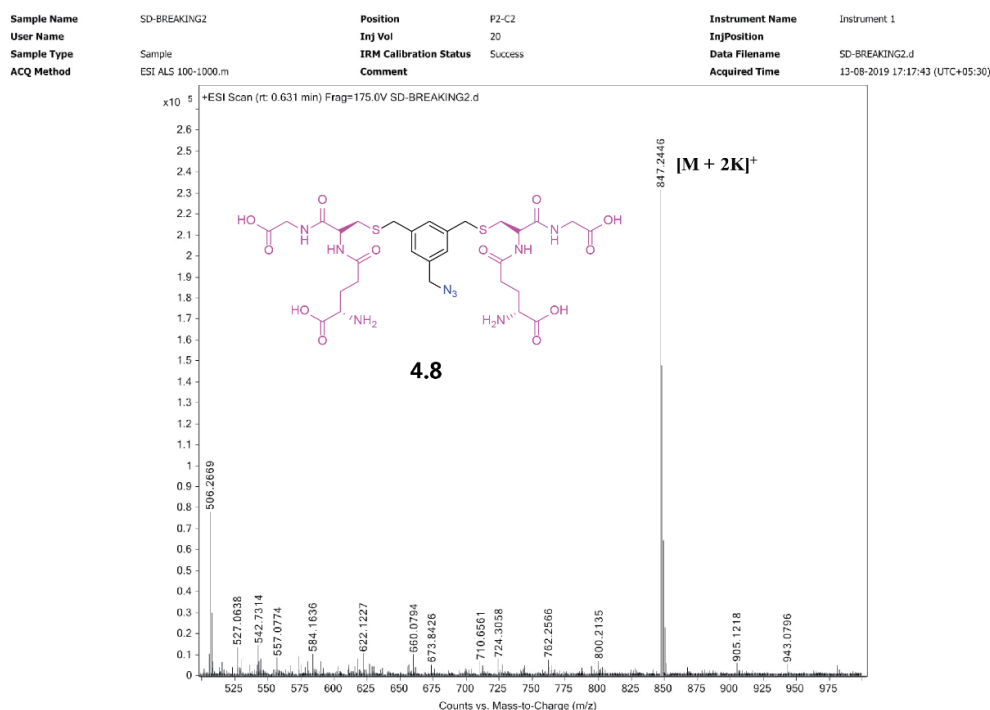


**Figure 3.20.** Dynamic light scattering (DLS) measurements. Size distribution of the extruded vesicles (200 nm) generated from **3.SL1a** lipid measured at 25 °C. Content is given in percentage distribution of different sized vesicles at different pH.

## 3.4.17. Mass spectrum analysis of the glutathione mediated reaction of the lipid

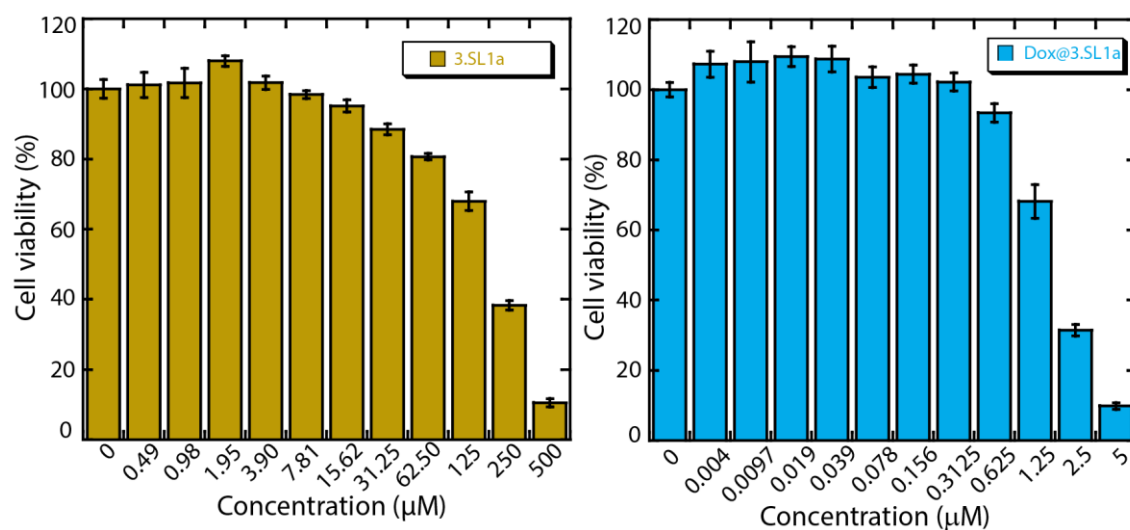


**Figure 3.21.** Mass spectral signals of compounds **3.7** (in water) after separation by HPLC analysis.



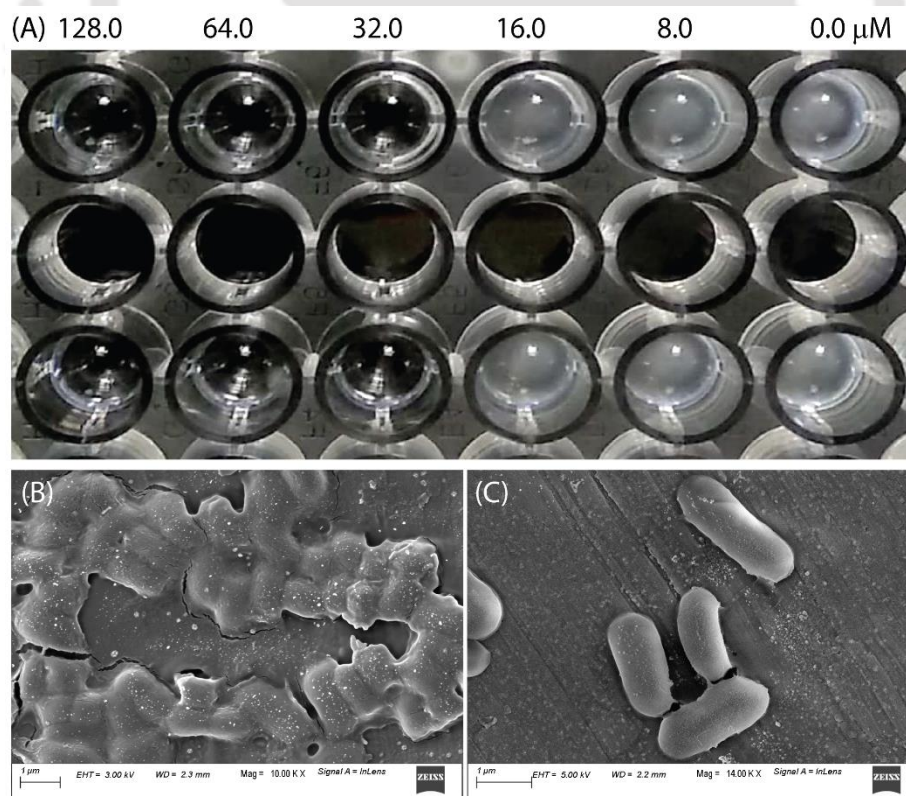
**Figure 3.22.** Mass spectral signals of compounds **3.8** (in water) after separation by HPLC analysis.

### 3.4.18. Cytotoxicity analysis



**Figure 3.23.** Viability of BJ cells in the presence of **3.SL1a** (C) and Dox@**3.SL1a** (D) at different concentrations. The cell viability were measured by MTT assay after 48 hours of compound treatment.

### 3.4.19. Antibacterial activity of the lipid against *E.coli*.



**Figure 3.24.** The 96 well plate bacterial assay of **3.SL1a** lipid (first and third row) against *E. coli* (MTCC-1687S). The clear wells specify inhibition of bacterial growth, while cloudy wells indicate unconstrained bacterial growth. Field emission scanning electron

microscope image of *E. coli* (MTCC-1687S) treated with only **3.SL1a** lipid (B). Field emission scanning electron microscope image of *E. coli* (MTCC-1687S) (C).

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## Chapter 4

### *Development of photoresponsive macrocyclic lipid for anti-cancer drug delivery*







#### 4.1. Background and objective of present work

Stimuli-responsive liposomes are prospective nanocarriers that deliver the therapeutic molecule at the target site, leading to therapeutic drug concentrations at the diseased site and reduced toxic effects in healthy tissues. Stimuli-responsive linkers are covalently attached to the lipid molecules that can be cleaved following delivery to the cells; this provides a controllable tool for triggering the release of encapsulated drug molecules. The endogenous stimuli are unique for diseased cells or tissue, and they can be exploited to augment the specificity of the drug molecules.<sup>1</sup> However, most of the cleavages mediated by these endogenous stimuli are restricted by the limiting conditions obligatory for the release, as they rely on differences in properties between cells. The use of a near-infrared (NIR; exogenous stimulus) light-responsive drug delivery system (DDS) is one of the attractive strategies as it can be used over long distances with excellent spatial and temporal resolutions.<sup>2-4</sup> Meanwhile, the poor biocompatibility of several synthetic cationic lipids restricted their clinical applications. Therefore, it is imperative to develop easily synthesizable and biocompatible photoresponsive lipids. Recently, a photoresponsive lipid-drug conjugate was developed to deliver drug molecules to cancer cells.<sup>5</sup> However, the use of UV-light restricted its further clinical applications.<sup>5,6</sup> Several liposome-based DDSs were also developed where cocktail mixtures of drug molecules and NIR-light-sensitive dyes were used for NIR-light-mediated drug delivery applications.<sup>7-9</sup> This approach is time-consuming and an economic burden for both pre-clinical and clinical validation of each molecule associated with the DDSs and the cocktail mixtures of agents. However, the use of only NIR-light-responsive lipids requires the clinical validation of a lower number of molecules. Most of the reported NIR-light responsive drug carriers are designed with the ester or carbamate moiety as the linker with the photon capturing 2-nitrobenzyl alcohol group. However, the direct attachment of ester, carbamate, and oxime functional groups to the 2-nitrobenzyl moiety makes the linker more chemically susceptible and enzymatic degradation.<sup>10,</sup>

11

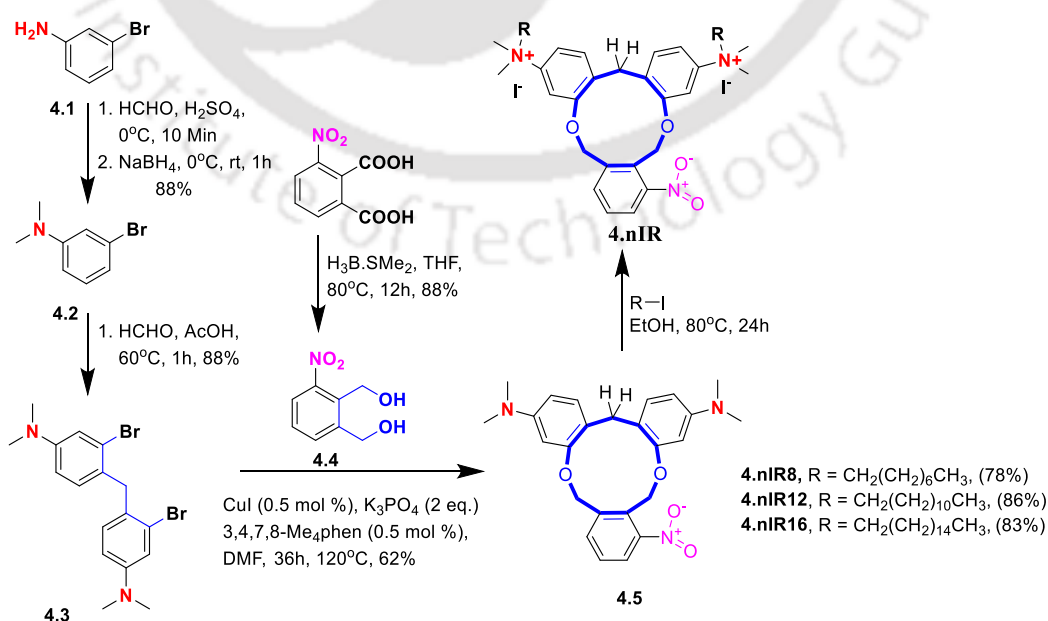
To curtail the aforementioned problems of structural complexity and linker instability, in this study, we demonstrated the synthesis of 2-nitrobenzyl containing 11-membered strained macrocyclic amphiphiles. We hypothesized that the installation of the UV-active, 2-nitrobenzyl moiety within the highly strained

macrocyclic moiety would make it NIR sensitive and endow it with substantial structural change, aggregation behavior, and efficient drug release behavior. Macrocyclic compounds such as calixarenes, cyclodextrins, and others have been used in drug and gene delivery,<sup>12</sup> but NIR-light-responsive macrocyclic amphiphiles have never been applied in drug delivery and represent a comprehensive strategy for smart DDSs. One of the synthesized gemini cationic amphiphile showed successful encapsulation and delivery of the most widely used hydrophobic chemotherapeutic drug Dox to cancer cells.

## 4.2. Results and discussions

### 4.2.1. Synthesis of photoresponsive macrocyclic lipids

Modular amphiphiles were synthesized from 3-bromoaniline (Scheme 4.1). The reductive methylation of 3-bromoaniline provided 3-bromo-*N,N*-dimethylaniline (**4.2**). The condensation of **4.2** with formaldehyde provided 4,4'-methylenebis(3-bromo-*N,N*-dimethylaniline) (**4.3**).<sup>13</sup> Subsequent ring-closure reaction *via* Ullmann-type C–O coupling reaction between **4.3** and (3-nitro-1,2-phenylene)dimethanol (**4.4**) resulted in the macrocyclic compound *N*<sup>3</sup>,*N*<sup>3</sup>,*N*<sup>14</sup>,*N*<sup>14</sup>-tetramethyl-7-nitro-6,11-dihydro-17*H*-tribenzo[*c,g,j*][1,6]dioxacycloundecine-3,14-diamine (**4.5**). Finally, *N*-alkylation of compound **4.5** with iodoctane, 1-iodododecane, and 1-iodohexadecane yielded compounds **4.nIR8**, **4.nIR12**, and **4.nIR16**, respectively.



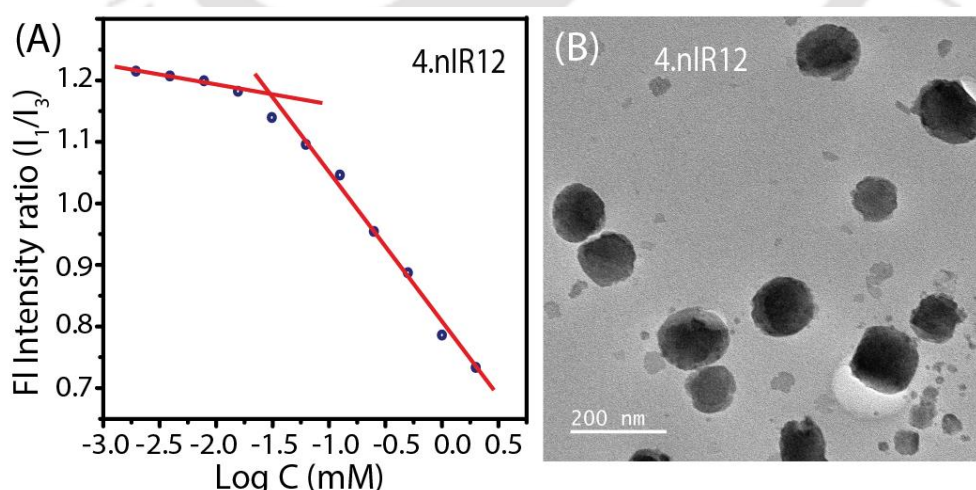
**Scheme 4.1.** Synthetic routes of the macrocyclic photoresponsive amphiphiles.

### 4.2.2. Critical aggregation concentration (CAC) of the lipid

The hydration of amphiphilic **4.nIR12** resulted in the spontaneous formation of colloidal aggregates. The hydrophobic effect could be the major driving force for its self-assembly in the aqueous medium. However, the other synthesized amphiphiles failed to self-assemble in the aqueous medium, which could be due to either the high hydration energy (**4.nIR8**) or the very low hydration energy (**4.nIR16**). The pyrene-based fluorescence assay revealed that the critical aggregation constant (CAC) of **4.nIR12** was 28.7  $\mu\text{M}$  (Figure 4.1). The lower CAC value suggests the tighter packing of **4.nIR12** molecules within the spherical NAs, which could be due to the presence of a conformationally restricted cyclic ring in the headgroup region.

### 4.2.3. Morphology of the lipid vesicles

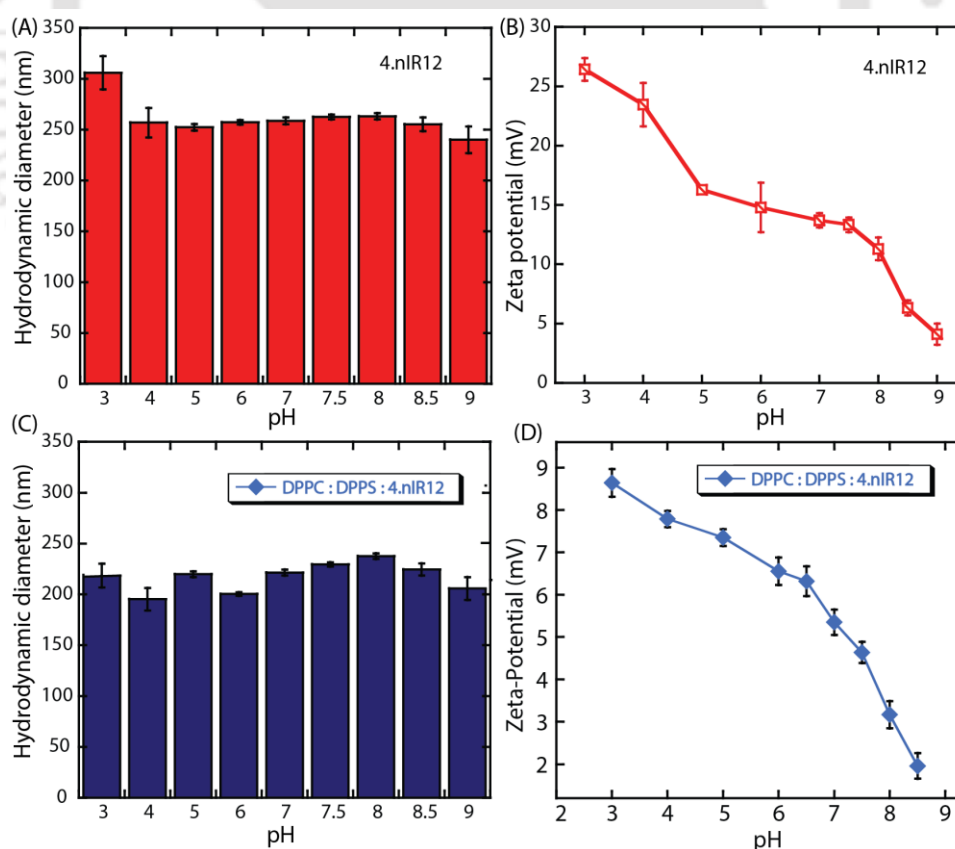
The morphology of the nanoaggregates (NAs) of lipid **4.nIR12** was investigated by transmission electron microscope (TEM). Inclusion of the lipid **4.nIR12** to a mixture of model lipids like 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2 Dipalmitoylsnglycero-3-phospho-l-serine (DPPS) (in the molar ratio of DPPC/DPPS/**4.nIR12** (2 : 2 : 1)) also showed excellent vesicle-formation capabilities (Figure 4.16). This indicates that the competence of macrocyclic amphiphiles in forming vesicles with other natural phospholipids, which could be advantageous in preparing size-tunable vesicles.



**Figure 4.1.** Measurement of the critical aggregation constant of **4.nIR12** using a pyrene probe (A). Representative FETEM image of the water-soluble aggregates produced from only **4.nIR12** (B).

#### 4.2.4. Zeta potential and hydrodynamic diameter of the lipid nanoaggregates

The pH-dependent dynamic light scattering (DLS) measurements showed that the hydrodynamic diameter of **4.nIR12** varies within 225–275 nm, suggesting its stability in different environments (Figure 4.2).<sup>14</sup> The smaller hydrodynamic diameter and pH-dependent stability of **4.nIR12** could be useful for its effective use in clinical applications due to the enhanced permeation and retention (EPR) effect.<sup>15</sup> The surface potential of the NAs was around +13.0 mV at physiological pH, which could be useful for selective targeting of cancer cells over normal cells (Figure 4.2). Meanwhile, the pH-dependent DLS and surface potential measurements of a DPPC/DPPS/**4.nIR12** (2:2:1) lipid mixture also showed that the hydrodynamic diameter varies within 225–250 nm, and the surface potential of the NAs was around +4.8 mV at physiological pH (Figure 4.2). Therefore, these cationic NAs could show preferential uptake efficiency by cancer cells.<sup>16, 17</sup> The decrease in surface potential with the increase in pH could be due to the increased solvation number.

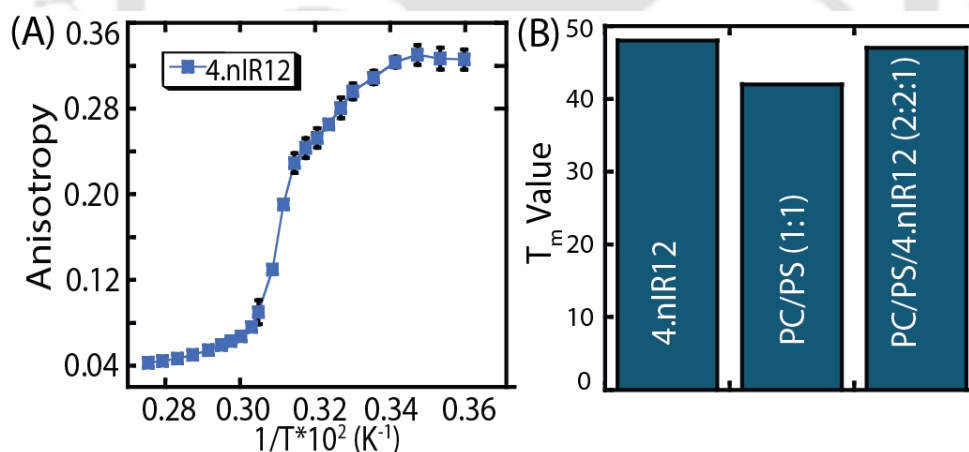


**Figure 4.2.** Variation of hydrodynamic diameter (A) and zeta potential (B) of the soluble aggregates of **4.nIR12** at various pH. Variation of hydrodynamic diameter (C)

and zeta potential (D) of the soluble aggregates of DPPC/DPPS/**4.nIR12** (molar ratio 2:2:1) at various pH.

#### 4.2.5. Phase transition temperature measurements

The temperature-dependent steady-state anisotropy measurements revealed that the phase transition temperature ( $T_m$ ) value of **4.nIR12** (48 °C; Figure 4.3) is much higher than that of the dipalmitoylphosphatidylcholine (DPPC) lipid (41 °C), demonstrating its higher thermal stability and superior drug retention capability at normal body temperature.<sup>17, 18</sup> The  $T_m$  value for a mixture of lipids DPPC and dipalmitoylphosphatidylcholine (DPPS) (1:1) was 42 °C. In comparison, the  $T_m$  value for the DPPC/DPPS/**4.nIR12** (2:2:1) lipid mixture was 47 °C (Figure 4.3) This increase of the  $T_m$  value could be due to the interaction of the cationic ammonium moieties of **4.nIR12** with the phosphate group of the phospholipids. The lipid mixture also showed excellent liposome formation capability, suggesting the ability of **4.nIR12** to form liposomes with other model lipids (Figure 4.3) which could be useful in drug delivery, preparing size controllable liposomes, and others.

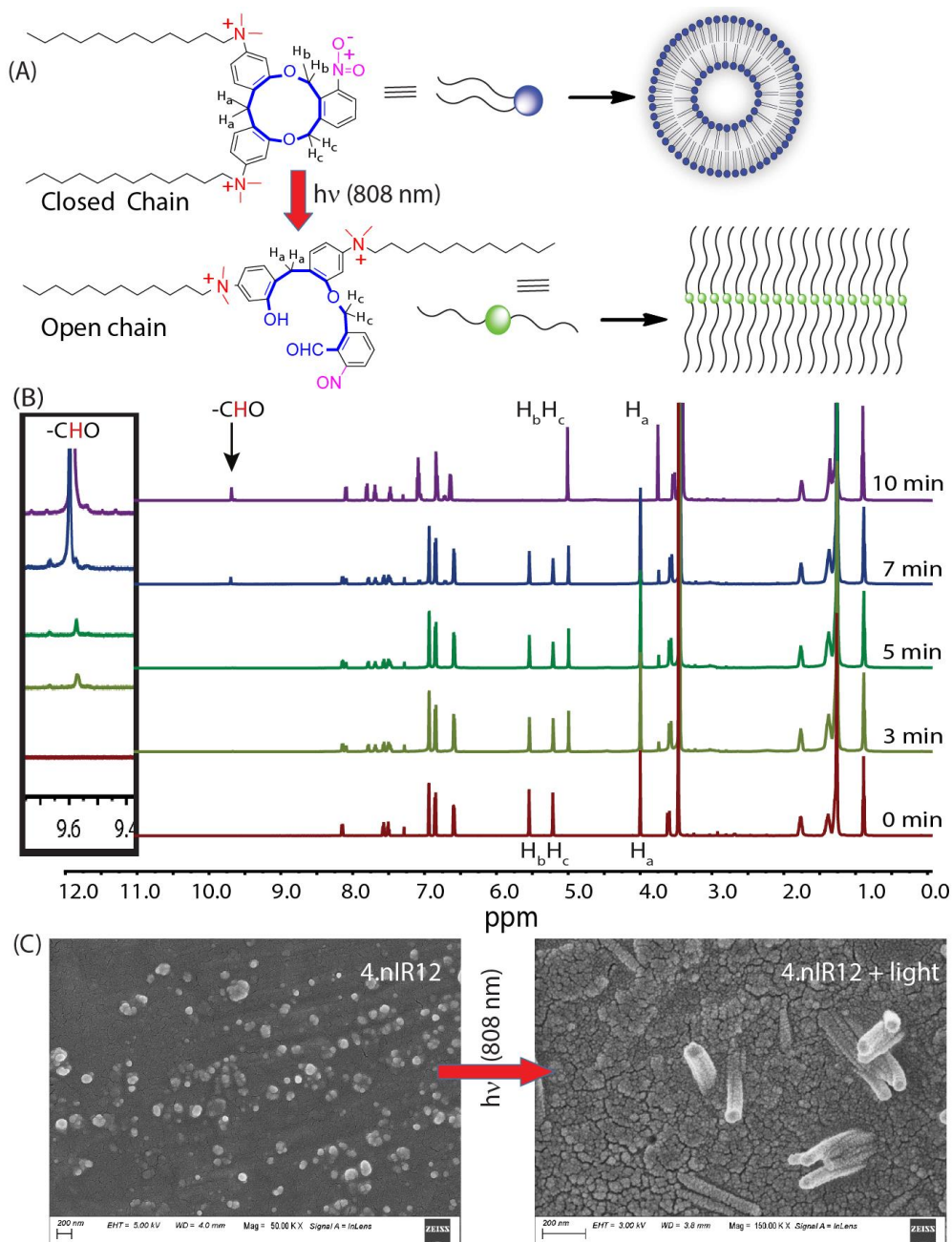


**Figure 4.3.** The temperature-dependent fluorescence anisotropy measurement of environment-sensitive 1,6-diphenyl-1,3,5-hexatriene dye incorporated within the water-soluble aggregates (A). Comparison study of phase transition temperature of lipid **4.nIR12**, DPPC/DPPS (1:1) and DPPC/DPPS/**4.nIR12** (2:2:1).

#### 4.2.6. NIR light induced photo cleavage of the compound

The NIR-light-mediated ring-opening (Figure 4.4A) of macrocyclic amphiphile **4.nIR12** was confirmed by  $^1\text{H}$  NMR (Figure 4.4B). A solution of **4.nIR12** in  $\text{CDCl}_3$  solvent was irradiated with NIR laser light ( $808\text{ nm}$ ,  $1\text{ W cm}^{-2}$ ) at different time intervals (0, 3, 5, 7, and 10 minutes). The time-dependent  $^1\text{H}$  NMR analyses revealed the disappearance of characteristic  $\text{H}_b$  proton signals (benzyl moiety) at  $\delta = 5.56\text{ ppm}$  and the appearance of a new signal at  $\delta = 9.69\text{ ppm}$  that corresponds to the aldehyde proton. In addition,  $\text{H}_a$  ( $\delta = 4.00\text{ ppm}$ ) and  $\text{H}_c$  ( $\delta = 5.22\text{ ppm}$ ) proton signals were also shifted to  $\delta = 3.78$  and  $5.01\text{ ppm}$ , respectively. Overall, these changes in proton signals in the  $^1\text{H}$  NMR spectra upon photoirradiation indicate the cleavage of the o-nitrobenzyl moiety and the opening of the 11-membered strained macrocyclic ring which was further confirmed by mass spectrometric analysis (Figure 4.16). The strained cyclic moiety of **4.nIR12** is linked with a photon absorbing 2-nitrobenzyl moiety. On NIR-light absorption, the 2-nitrobenzyl group depletes the energy by opening the constrained ring to an open-chain conformation. Within the macrocyclic system, the molecular angular strain leads to a drift from the tetrahedral geometry, which is liable to easy cleavage of the C–O bond via the Norrish type II mechanism.<sup>19</sup> The FESEM and FETEM analysis of **4.nIR12** in the presence of NIR-light also showed a significant change in its aggregation patterns, indicating its instability upon photoirradiation. The spherical aggregation pattern of **4.nIR12** in the aqueous medium turned into nanotubular self-assembly (diameter around  $3.7\text{ nm}$ ) upon NIR irradiation (Figure 4.4C) which could allow the release of encapsulated drug molecules. The photocleavage of the 2-nitrobenzyl containing macrocyclic ring generated an open-chain conformation, which would self-aggregate in the aqueous medium to form the nanotubular assembly. These organic nanotubes could be beneficial for constructing building blocks for various optoelectronic devices.<sup>20</sup> The FETEM analysis of the DPPC/ DPPS/**4.nIR12** lipid mixture showed complete disruption of the spherical self-assembly after the NIR-light treatment (Figure 4.17).





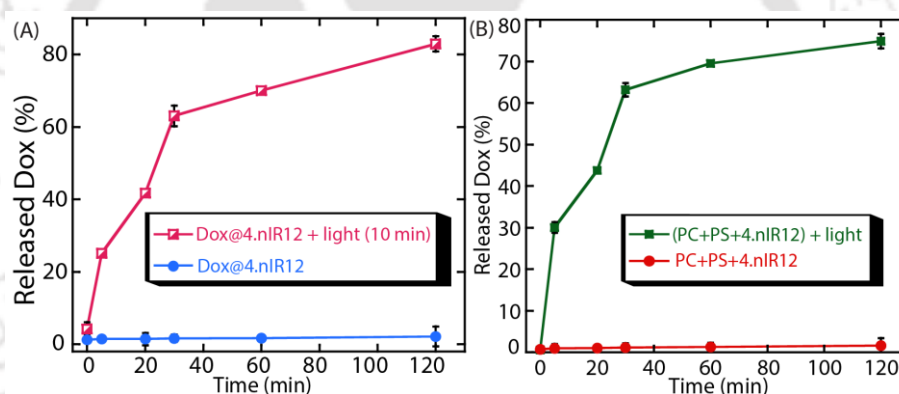
**Figure 4.4.** The schematic diagram represents the self-aggregation patterns of **4.nIR12** before and after NIR-light treatment (10 min) (A).  $^1\text{H}$  NMR spectra of **4.nIR12** without and with NIR-light treatment at different time intervals (B). Representative FESEM images of the water-soluble aggregates produced only **4.nIR12** before and after NIR-light treatment (10 min) (C).

#### 4.2.7. Drug loading and release efficiency of vesicle of **4.nIR12** lipid

NIR light-mediated deformation of the NAs of lipid **4.nIR12** encouraged us to investigate the drug loading and release profile. The only **4.nIR12** and



DPPC/DPPS/**4.nIR12** lipid mixture showed adequate loading capability of hydrophobic Dox molecules (41 and 46% at pH 7.4) for the chemotherapeutic drug, Dox, suggesting that the hydrophobic Dox molecules could be encapsulated within the hydrophobic core of the spherical assembly of **4.nIR12**.<sup>14</sup> The Dox release profiles without and with photoirradiation (10 min) showed 2% and 83% release efficiencies after 2 h, respectively (Figure 4.5A), suggesting the conformational changes in the presence of NIR light led to the destabilization of the bilayer assembly of **4.nIR12** and induced Dox release capability. The Dox release efficiencies from the lipid mixture without and with photoirradiation (10 min) were 2% and 74% after 2 h, respectively (Figure 4.5B). This faster Dox release profile of **4.nIR12** is beneficial for its drug delivery applications, including minimizing adverse effects because of the nonspecific delivery to the healthy cells and augmentation of the local concentration of the Dox molecules within the cancer cells and others.



**Figure 4.5.** The Dox release profiles of **4.nIR12** at pH 7.4 without and with NIR-light treatment (10 min) (A). The Dox release profile of DPPC/DPPS/**4.nIR12** (2:2:1) at pH 7.4 without and with NIR-light treatment (10 min).

## 4.2.8. Cell viability and cellular uptake analysis

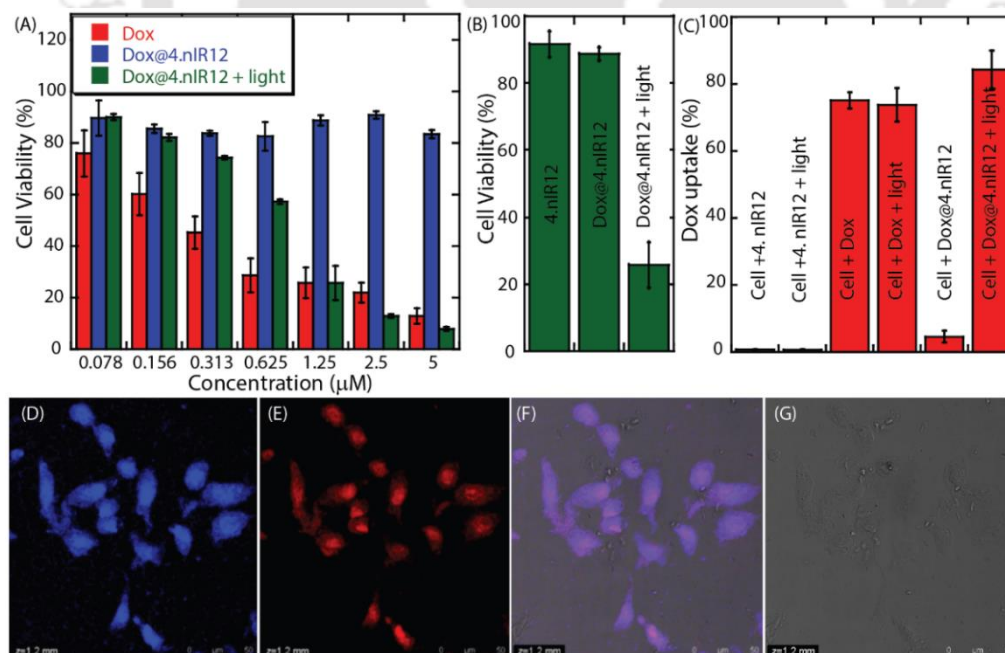
### 4.2.8.1. Cell Viability

The cell viability assay showed that **4.nIR12** had a low or negligible toxic effect (after 48 h of incubation) on human MDA-MB- 231 (triple-negative breast cancer) cells and human peripheral blood mononuclear cells (PBMCs; healthy cells), indicating its significance in drug delivery applications (Figure 4.6 and 4.18).<sup>21</sup> The MTT assay showed that the  $IC_{50}$  value of free Dox was  $0.23 \pm 0.02 \mu M$ . The MTT assay revealed that the  $IC_{50}$  value of Dox encapsulated **4.nIR12** (Dox@**4.nIR12**) with NIR-light treatment (10 min) was  $0.72 \pm 0.05 \mu M$  (with respect to the effective Dox

concentration). However, when the cells were incubated with Dox@4.nIR12 for 48 h without NIR-light treatment, no significant cytotoxicity was observed (Fig. 4.6A and B). Hence, the MTT assay results support the successful entrapment and photorelease efficiencies of Dox molecules.

#### 4.2.8.2. Cellular uptake

The cell internalization of the drug loaded vehicle was analyzed by flow cytometry and confocal laser scanning microscopy (CLSM).<sup>14, 22</sup> The flow cytometry analysis also showed effectual cellular uptake efficiency (Figure 4.6C and 4.19 ). The CLSM images revealed the efficient Dox delivery and release efficacy of 4.nIR12 in the presence of NIR-light (Figure 4.6E). The high Dox@4.nIR12 uptake efficiency could be because of the preferable electrostatic interaction of the gemini cationic amphiphile with the negatively charged lipids present in higher abundance on the outer membrane of cancerous MDA-MB-231 cells, followed by the endocytosis process. This directed electrostatic interaction could also reduce the side effects of the Dox molecules due to nonspecific delivery.<sup>23</sup> Hence, the high cellular uptake efficacy of the Dox molecules indicates that this exogenous stimulus significantly controls the drug release efficiency of 4.nIR12.



**Figure 4.6.** Viabilities of MDA-MB-231 cells in the presence of free Dox and Dox@4.nIR12 after 48 h of incubation (A). Viabilities of MDA-MB-231 cells in the

presence of **nIR12** (125  $\mu\text{M}$ ) and Dox@**4.nIR12** (1.25  $\mu\text{M}$  Dox and 125  $\mu\text{M}$  amphiphile) (B). FACS analysis for Dox uptake efficiencies of MDA-MB-231 cells treated with **4.nIR12**, free Dox (125  $\mu\text{M}$ ), and Dox@**4.nIR12** (1.25  $\mu\text{M}$  Dox and 125  $\mu\text{M}$  **4.nIR12**) (C). CLSM images of the MDA-MB-231 cells incubated with Dox@**nIR12** (1.25  $\mu\text{M}$  Dox and 125  $\mu\text{M}$  amphiphile) for 8 h followed by NIR-light treatment for 10 min. The blue channel (DAPI; D), red channel (Dox; E), overlay of the blue and red channels (F), and bright-field (G) illustrate the Dox release efficacy to MDA-MB-231 cells. Scale bars: 50  $\mu\text{m}$ . The NIR-light (808 nm, 1 W  $\text{cm}^{-2}$ ) treatment was done for 10 min.

### 4.3. Summary

we developed an o-nitrobenzyl containing macrocyclic gemini cationic amphiphile that self-assembles in an aqueous environment to form soluble spherical NAs with favorable properties of DDSs. The NIR-responsive cleavage of the o-nitrobenzyl moiety led to the opening of the strained dioxacycloundecine ring to generate a nanotubular self-assembly in the aqueous environment could be the driving force for the release of encapsulated drug molecules. The amphiphile showed lower cytotoxicity and successful entrapment and delivery of the hydrophobic anticancer drug Dox to cancerous MDA-MB-231 cells. The NIR-light mediated photocleavage of the amphiphile within the cancer cells promotes cell death. Hence, this simple and innovative concept of developing macrocyclic gemini cationic amphiphiles as potential DDSs could be useful in photodynamic therapy, which could be dissected in a controlled fashion using NIR-light to liberate the enveloped hydrophobic chemotherapeutic drug molecules in a spatially- and temporally-controlled manner for surface and deep-tissue cancer treatment.

### 4.4. Experimental section

#### 4.4.1. Synthesis of the compounds

##### 4.4.1.1. General information

The reagents were procured from Sigma-Aldrich, Merck, and other commercial sources and used directly without further purification. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 (0.25 mm). The column chromatography was performed using 60–120 mesh silica gels. The  $^1\text{H}$  NMR and  $^{13}\text{C}$

NMR were recorded at 600 and 151 MHz with a Varian AS400 spectrometer and Bruker spectrometer, respectively. The chemical shifts were reported in parts per million ( $\delta$ ) using DMSO- $d_6$ , CDCl<sub>3</sub> as the internal solvent. The coupling constants ( $J$  values) and chemical shifts ( $\delta_{\text{ppm}}$ ) were reported in Hertz (Hz) and parts per million (ppm), respectively, downfield from tetramethylsilane using residual chloroform ( $\delta$  = 7.28 ppm for  $^1\text{H}$  NMR,  $\delta$  = 77.23 ppm for  $^{13}\text{C}$  NMR) as an internal standard. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broadened). High-resolution mass spectra (HRMS) were recorded at Agilent Q-TOF mass spectrometer with Z-spray source using built-in software for analysis of the recorded data. Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers. The stock solutions of compounds were prepared in gas chromatographic grade DMSO which was also purchased from Sigma.

#### 4.4.1.2. General procedure for the synthesis of targeted compounds

##### 4.4.1.2.1. Synthesis of 3-bromo-*N,N*-dimethylaniline (4.2)

To a stirring solution of aqueous H<sub>2</sub>SO<sub>4</sub> (3 M, 3.5 ml) and aqueous formaldehyde (37%, 1.30 mL, 17.43 mmol) in tetrahydrofuran (15 mL) at 0 °C was added 3-bromoaniline (1.0 g, 5.8 mmol), and the reaction mixture was stirred for 10 min. Then, solid NaBH<sub>4</sub> (0.88 mg, 23.25 mmol) was slowly added, and the temperature was maintained at 0 °C. After that, the resulting reaction mixture was allowed to warm up and stirred for 1 h at 20 °C. After maximum consumption of the starting materials, the reaction mixture was diluted with a saturated aqueous solution of NaHCO<sub>3</sub> (20 mL), and the reaction mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 20 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and filtered, and solvents were evaporated under reduced pressure. The yellow crude product was of sufficient purity for the next step (yield: 1.173; 88%). The  $^1\text{H}$  and  $^{13}\text{C}$  NMR of the compound is in accordance with the literature report.<sup>13</sup>

##### 4.4.1.2.2. Synthesis of 4,4'-methylenebis(3-bromo-*N,N*-dimethylaniline) (4.3)

To a stirring solution of 3-bromo-*N,N*-dimethylaniline (4.2, 1.0 g, 4.97 mmol) in glacial acetic acid (10 mL) aqueous formaldehyde (37%, 0.19 mL, 2.48 mmol) was added dropwise over 15 min. The reaction mixture was then stirred at 90 °C for 1-

hour, and acetic acid was evaporated under reduced pressure. The residual acid was then neutralized with a saturated aqueous solution of  $\text{NaHCO}_3$ . After that, the reaction mixture was extracted with  $\text{CH}_2\text{Cl}_2$  ( $3 \times 20$  mL). The combined organic layers were washed with water ( $2 \times 20$  mL) and brine (30 mL), dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and filtered. The solvents were removed under reduced pressure. The resulting red liquid was purified through silica gel column chromatography using a gradient solvent system of ethyl acetate in hexane (0 – 10%) to provide the pure product as a white crystalline solid (yield: 0.74 mg; 88%). The  $^1\text{H}$  and  $^{13}\text{C}$  NMR of the compound is in accordance with the literature report.<sup>13</sup>

#### 4.4.1.2.3. Synthesis of (3-nitro-1,2-phenylene)dimethanol (4.4)

To a stirring solution of 3-nitrophthalic acid (500 mg, 2.36 mmol) in dried THF was added 10 M solution of borane dimethylsulfide complex (dropwise addition; 2.5 equiv.). Then, the resulting solution was heated at 80 °C for 12 hours. After that, a 3 M aqueous solution of hydrochloric acid was added to this reaction mixture (dropwise) until effervescence was no longer observed. The resulting mixture was then extracted with  $3 \times 20$  mL of ethyl acetate. The combined organic layers were washed with saturated aq.  $\text{NaHCO}_3$  is followed by brine. The resulting organic phase was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and filtered. The filtrate was concentrated under reduced pressure to afford the crude mixture. The mixture was purified through silica gel column chromatography with a solvent gradient system of 5–15% ethyl acetate in hexane to afford the pure compound **4.4**. Characterization of the compound: Colorless solid (yield: 88%)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  8.20 – 8.14 (m, 1H), 7.59 – 7.50 (m, 2H), 5.55 (s, 2H), 5.22 (s, 2H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  143.0, 136.3, 129.0, 127.0, 122.7, 74.0, 73.2; HRMS (ESI) calcd. for  $\text{C}_8\text{H}_9\text{NO}_4$   $[\text{M} + \text{K}]^+$ : 222.0169, found 222.0209.

#### 4.4.1.2.4. Synthesis of $N^3, N^3, N^{14}, N^{14}$ -tetramethyl-7-nitro-6,11-dihydro-17H-tribenzo[*c,g,j*][1,6]dioxacycloundecine-3,14-diamine (4.5)

Compound **4.3** (500 mg, 1.21 mmol), (3-nitro-1,2-phenylene)dimethanol (489 mg, 2.66 mmol),  $\text{K}_3\text{PO}_4$  (1.1g, 5.32 mmol),  $\text{CuI}$  (115 mg, 0.65 mmol,) and 3,4,7,8-tetramethyl-1,10-phenanthroline (142 mg, 0.65 mmol) were added to a screw capped seal tube under argon atmosphere. The tube was then evacuated and



backfilled with nitrogen for three times. Then dry DMF (4 mL) was added by syringe at room temperature. The reaction mixture was stirred at 120°C for 24 h. The reaction mixture was allowed to reach room temperature and then diluted with ethylacetate (20 mL). The slurry was filtered, and filter cake was washed with 10 mL of dichloromethane. The organic layers were removed under reduced pressure, and the residue was purified by column chromatography on silica gel using a gradient solvent system of 0 – 10% ethyl acetate in hexane to afford the desired compound **4.5**. Characterization of the compound: Colorless solid (yield: 62%)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  8.17 – 8.16 (m, 1H), 7.59 – 7.58 (m, 1H), 7.53 – 7.50 (s, 1H), 6.96 (s, 2H), 6.89 – 6.86 (m, 2H), 6.62 – 6.60 (m, 2H), 5.57 (s, 2H), 5.23 (s, 2H), 4.02 (s, 2H), 2.94 (s, 12H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  154.0, 143.0, 136.3, 130.7, 129.0, 127.0, 125.6, 122.7, 116.2, 111.8, 74.8, 73.2, 40.5, 39.9; HRMS (ESI) calcd. for  $\text{C}_{25}\text{H}_{27}\text{N}_3\text{O}_4$   $[\text{M} + \text{H}]^+$  : 434.2074, found: 434.2076.

#### 4.4.1.2.5. Synthesis of $N^2,N^2,N^{15},N^{15}$ -tetramethyl-7-nitro- $N^2,N^{15}$ -dioctyl-6,11-dihydro-17H-tribenzo[*c,g,j*][1,6]dioxacycloundecine-2,15-diaminium (**4.nIR8**)

To a stirring solution of compound **4.5** was added  $\text{K}_2\text{CO}_3$  (0.827 mmol), and the reaction mixture was continued to stir for a few minutes at room temperature. After that, 1-iodooctane (1.74 mmol) was added, and the reaction mixture was heated under reflux conditions for 12 hours. The progress of the reaction was monitored by TLC. After maximum consumption of compound **4.5**, the reaction mixture was cooled down to room temperature, and unused  $\text{K}_2\text{CO}_3$  was removed by filtration. Then the organic solvent was removed under reduced pressure to yield an oily crude product. The mixture was purified through silica gel column chromatography with a solvent gradient system of 15–35% ethyl acetate in hexane to afford the pure product, **4.nIR8**. Characterization of compound **4.nIR8**: brown gummy liquid (yield: 78%)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  8.17 – 8.15 (m, 1H), 7.59 – 7.58 (m, 1H), 7.53 – 7.51 (s, 1H), 6.95 (s, 2H), 6.88 – 6.86 (m, 2H), 6.61 – 6.59 (m, 2H), 5.56 (s, 2H), 5.23 (s, 2H), 4.01 (s, 2H), 3.62 – 3.59 (m, 4H), 3.48 (s, 12H), 1.79 – 1.75 (m, 4H), 1.41 – 1.36 (m, 4H), 1.31 – 1.27 (m, 23H), 0.90 (t, 6H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  154.0, 143.0, 136.3, 130.7, 129.0, 127.0, 125.6, 122.7, 116.2, 111.8, 74.8, 73.2, 67.3, 53.7, 40.5, 39.8, 31.9, 29.6, 29.4, 29.4, 29.3, 26.0, 23.2, 22.7, 14.1; ESI-MS (ESI+)  $m/z$ :  $[(\text{M} + \text{HCOO}^-)]^+$  704.2220.

**4.4.1.2.6. Synthesis of  $N^2,N^{15}$ -didodecyl- $N^2,N^2,N^{15},N^{15}$ -tetramethyl-7-nitro-6,11-dihydro-17*H*-tribenzo[*c,g,j*][1,6]dioxacycloundecine-2,15-diaminium (4.nIR12)**

To a stirring solution of compound **4.5** was added  $K_2CO_3$  (0.827 mmol) and the reaction mixture was continued to stir for few minutes at room temperature. After that, 1-iodododecane (1.74 mmol) was added, and the reaction mixture was heated under reflux condition for 12 hours. The progress of the reaction was monitored by TLC. After maximum consumption of compound **4.5**, the reaction mixture was cooled down to room temperature, and unused  $K_2CO_3$  was removed by filtration. Then organic solvent was removed under reduced pressure yielded an oily crude product. The mixture was purified through silica gel column chromatography with a solvent gradient system of 15–30% ethyl acetate in hexane to afford the pure product, **4.nIR12**. Characterization of compound **4.nIR12**: Brown gummy liquid (yield: 86%)  $^1H$  NMR (600 MHz,  $CDCl_3$ )  $\delta_{ppm}$  8.16 – 8.14 (m, 1H), 7.58 – 7.57 (m, 1H), 7.52 – 7.51 (s, 1H), 6.94 – 6.93 (m, 2H), 6.86 – 6.85 (m, 2H), 6.60 – 6.58 (m, 2H), 5.55 (s, 2H), 5.22 (s, 2H), 4.00 (s, 2H), 3.62 – 3.59 (m, 4H), 3.47 (s, 12H), 1.78 – 1.75 (m, 4H), 1.40 – 1.38 (m, 4H), 1.31 – 1.26 (m, 23H), 0.89 (t, 6H);  $^{13}C$  NMR (151 MHz,  $CDCl_3$ )  $\delta_{ppm}$  150.0, 143.0, 136.3, 130.7, 129.0, 127.0, 125.6, 122.7, 116.2, 111.8, 74.8, 73.2, 67.3, 53.7, 40.5, 39.8, 31.9, 29.6, 29.4, 29.4, 29.3, 26.0, 23.2, 22.7, 14.1; ESI-MS (ESI+)  $m/z$ : [(M + 2HCOO) $^-$ ] $^+$  861.2861.

**4.4.1.2.7. Synthesis of  $N^2,N^{15}$ -dihexadecyl- $N^2,N^2,N^{15},N^{15}$ -tetramethyl-7-nitro-6,11-dihydro-17*H*-tribenzo[*c,g,j*][1,6]dioxacycloundecine-2,15-diaminium (nIR16)**

To a stirring solution of compound **4.5** was added  $K_2CO_3$  (0.827 mmol), and the reaction mixture was continued to stir for a few minutes at room temperature. After that, 1-iodohexadecane (1.74 mmol) was added, and the reaction mixture was heated under reflux conditions for 12 hours. The progress of the reaction was monitored by TLC. After maximum consumption of compound **4.5**, the reaction mixture was cooled down to room temperature, and unused  $K_2CO_3$  was removed by filtration. Then the organic solvent was removed under reduced pressure yielded an oily crude product. The mixture was purified through silica gel column chromatography with a solvent



gradient system of 15–30% ethyl acetate in hexane to afford the pure product as a brown gummy liquid, **4.nIR16**. Characterization of compound **4.nIR16**: Brown gummy liquid (yield: 83%)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  8.15 – 8.13 (m, 1H), 7.58 – 7.56 (m, 1H), 7.51 – 7.49 (s, 1H), 6.95 – 6.91 (m, 2H), 6.85 – 6.84 (m, 2H), 6.59 – 6.58 (m, 2H), 5.54 (s, 2H), 5.21 (s, 2H), 3.99 (s, 2H), 3.59 – 3.56 (m, 4H), 3.45 (s, 12H), 1.80 – 1.75 (m, 4H), 1.42 – 1.38 (m, 4H), 1.30 – 1.26 (m, 23H), 0.88 (t, 6H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  154.0, 143.0, 136.3, 130.8, 129.0, 127.1, 125.6, 122.7, 116.2, 111.8, 74.7, 73.2, 67.3, 53.9, 49.4, 40.5, 39.8, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 26.1, 23.2, 22.7, 14.1; ESI-MS (ESI+)  $m/z$ :  $[\text{M}]^+$  884.6778.

#### 4.4.2. Preparation of soluble aggregates

The compounds were dried under reduced pressure for 3 hours to prepare thin films. After that, 500  $\mu\text{L}$  of 20 mM HEPES buffer, pH 7.2, containing 100 mM KCl, was added to the thin film and heated to 30–40  $^\circ\text{C}$  for 10 min. The solution was then vortexed well to mix the thin film. Finally, the solution was sonicated more than ten times with 30 s of sonication followed by 30 s of cooling on ice cycles. A hand-held mini extruder with a polycarbonate membrane (diameter of 200 nm) was used to prepare small soluble aggregates at room temperature.

#### 4.4.3. Transmission electron microscopy analysis

The morphology of the aqueous soluble aggregates of the amphiphile was investigated both in the presence and absence of NIR (808 nm, 1  $\text{W cm}^{-2}$  for 10 min.) light by transmission electron microscope (TEM).<sup>14, 24</sup> First, the aqueous solution of the compound was prepared by the above-mentioned method in 20 mM HEPES buffer, pH 7.2, containing 100 mM KCl. A 10  $\mu\text{L}$  of the solution was placed onto a carbon-coated copper grid and allowed to settle down for 1 minute. The grid was then carefully blotted with filter paper, and only a trace amount of the solution in the middle of the grid was kept. After that, the grid was allowed to dry for 10 minutes at 30  $^\circ\text{C}$ . Finally, 10  $\mu\text{L}$  of 2% uranyl acetate solution (in water) was added to the grid and allowed to dry for another 1 min at room temperature. The excess uranyl acetate solution was soaked off with tissue paper, and the grid was dried overnight at 30  $^\circ\text{C}$ . The images were collected using JEOL JEM 2100 transmission electron microscope (operated at a maximum accelerating voltage of 200 kV).

#### 4.4.4. Phase transition temperature measurements

To determine the phase transition temperature ( $T_m$ ) of the aqueous soluble nanoaggregates, the temperature-dependent steady-state anisotropy measurements were performed.<sup>14</sup> The environment-sensitive dye 1, 6 -diphenyl-1, 3, 5-hexatriene (DPH) was used as the fluorescence probe to measure the change in anisotropy values. The soluble aggregates were prepared according to the method described in the previous section (the hydration was done by using 20 mM HEPES buffer, pH 7.2, containing 100 mM KCl). After that, the solution of compound (1000  $\mu$ L) and DPH solution (10  $\mu$ L of 1 mM stock solution in THF) was taken in a microcentrifuge tube (final concentration of DPH was 10  $\mu$ M; DPH < 1% v/v in compound solution). The solution was kept at room temperature under tumbling conditions for overnight to ensure the maximum incorporation of DPH molecules inside the hydrophobic core of the soluble aggregates. Then steady-state fluorescence anisotropy measurements were performed with a refrigerated system using a Peltier temperature controller connected to a Fluoromax-4 spectrofluorometric (Horiba Scientific). The degree ( $r$ ) of anisotropy in the DPH fluorescence ( $\lambda_{ex}$  = 350 nm;  $\lambda_{em}$  = 429 nm) was calculated using the following equation (eq. 1) at the peak of the fluorescence spectrum. The  $I_{VV}$  and  $I_{VH}$  are the fluorescence intensities of the emitted light polarized parallel and perpendicular to the excited light, respectively, and  $G = I_{VH}/I_{HH}$  is the instrumental grating factor.

$$r = \frac{(I_{VV} - G I_{VH})}{(I_{VV} + 2G I_{VH})} \dots\dots\dots \text{Eq. 4.1}$$

At every 3-4  $^{\circ}$ C temperature difference, the anisotropy values were recorded, apart from the near to the  $T_m$  value; in that case, the anisotropy values were collected at every 1 or 2  $^{\circ}$ C. All anisotropy values of the DPH probe are the mean values of three individual measurements. Finally, to determine the  $T_m$  value of the compound, plots of the degree of anisotropy ( $r$ ) of the DPH probe as a function of temperature were investigated.

#### 4.4.5. pH-dependent particle size and surface charge of the soluble aggregates

The soluble aggregates were prepared according to the method as described in the earlier section in 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl.<sup>14</sup> Different buffer solutions were freshly prepared using isosmotic buffers consisting of 10 mM buffering agent and 10 mM salt to vary the pH range (pH 3 to pH 9.0) and used for the measurements. Citric acid and trisodium-citrate for pH 3.0–6.5; 3-(*N*-morpholino)propanesulfonic acid (MOPS) for pH 7.0 and tris(hydroxymethyl)aminomethane (Tris)-HCl were used for pH 7.5–8.5. Both the zeta potential and hydrodynamic diameter of the soluble aggregates at different pH were measured using a Zetasizer Nano ZS90 (Malvern, Westborough, MA) instrument at 25 °C.

#### 4.4.6. NIR light-induced cleavage of the amphiphile

We monitored the light cleavage ability of the compound with the help of NMR. The compound **4.nIR12** was dissolved in CDCl<sub>3</sub>, and a NIR laser diode (808 nm) of power density (1 W cm<sup>-2</sup>) was set at 3 cm above the liquid surface and irradiated for 2, 3, 5, 7, 10 minutes, and the NMR spectra were recorded of corresponding time. The cleavage of **4.nIR12** was also analyzed in mass spectrometry.

#### 4.4.7. Doxorubicin encapsulation and release efficiency measurement

The DOX release profiles from vesicles were determined using dialysis-based method according to the reported procedure.<sup>25</sup> Vesicles containing DOX (1 mM DOX in 50 mM of lipid in HEPES buffer) were added into a D-Tube™ Dialyzer (Millipore Sigma, molecular weight cut-off 10 kD). Tubes were sealed and placed into a reservoir loaded with 10 mL of the same buffer. At scheduled time points, 20 µL of the reservoir solution was injected through analytical HPLC (C18, 15 cm × 4.6 mm, 2.7 µm) to monitor the release of free DOX. Concentrations of released DOX in the supernatant were determined by measuring area of the chromatogram at 254 nm. Acetonitrile and water were used as mobile phase with flow rate of 0.5 mL/minute. To measure the loaded drug, the DOX@**4.nIR12** and DOX@(PC/PS/**4.nIR12**) was dissolved in acetonitrile and recorded through HPLC.

#### 4.4.8. Cell culture

The MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and penicillin-streptomycin solution (GIBCO; 100 U/ml penicillin and 100 µg/ml streptomycin).<sup>14</sup> PBMC (Peripheral Blood Mononuclear Cell) were isolated from the blood sample of a healthy donor and cultured in DMEM supplemented with the above-mentioned media. Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C in a CO<sub>2</sub> incubator. In all experiments, the medium was changed daily with complete media.

#### 4.4.9. Determination of cell viability using MTT assay

The MDA-MB-231 and PBMC cells were cultured, as mentioned earlier.<sup>14</sup> For the cell viability study, both the cell lines were seeded in 96-well flat-bottomed plates at a density of  $2 \times 10^4$  cells per well and incubated for 24 hours in complete media in a CO<sub>2</sub> incubator. Thereafter, cells were treated with different concentrations of **4.nIR12**, free Dox, and Dox@**4.nIR12** (in the presence and absence of 808 nm light according to the experiment) for additional 48 hours. The cell viability was then assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma-Aldrich) assays. Briefly, media was discarded, and cells were treated with MTT (final concentration: 0.5 mg/ml) for 2 hours at 37 °C in a CO<sub>2</sub> incubator, and the resulting purple-colored formazan crystals were dissolved in 200 µL of DMSO. Cell viability was then measured using Multiscan GO (Thermo Scientific) at 570 nm. Cell viability (%) = (absorbance intensity (sample)) / (absorbance intensity (control)) × 100%.

#### 4.4.10. Determination of cellular uptake efficiency using flow cytometry

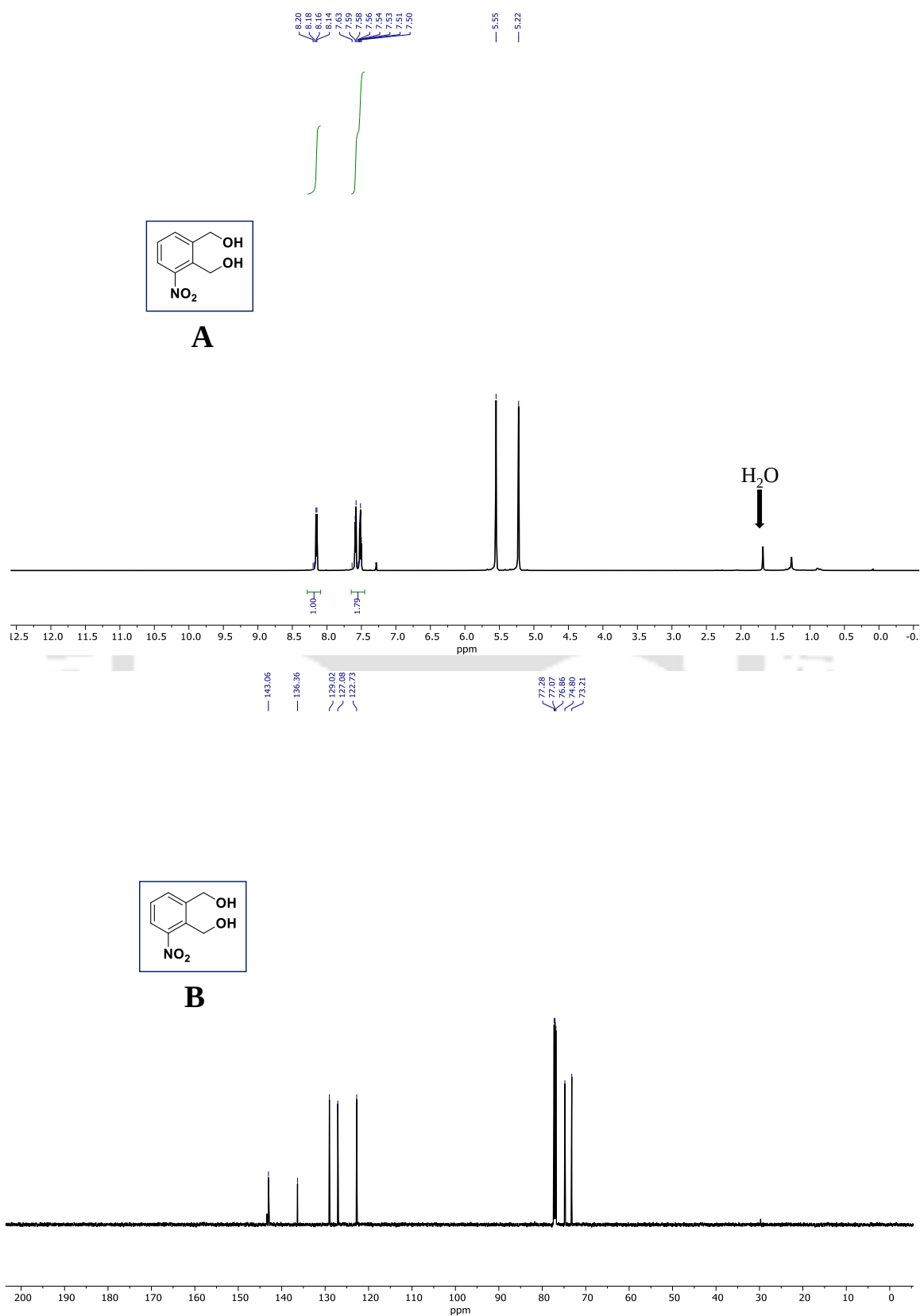
The MDA-MB-231 cells were cultured, as mentioned earlier.<sup>14</sup> Cells were seeded in 6-well plates at a density of  $2 \times 10^5$  cells per well and incubated for 24 hours in complete media. The cells were then treated with different entities (Dox, Dox@**4.nIR12**) in the absence and presence of NIR light (808 nm, 1 W cm<sup>-2</sup>, 10 min.) in 1% FBS containing media for 8 hours at 37 °C. Next, the cells were washed twice with PBS (GIBCO), trypsinized (0.05%; GIBCO) for 2 minutes at 37 °C followed by 5 min centrifugation at 800 rpm at 4°C. The supernatant was discarded, and cell pellets were re-suspended in 300 µL of PBS (supplemented with 2% FBS). Finally, the solutions were kept on ice, and the drug uptake by the MDA-MB-321 cells was

analyzed by flow cytometry analysis using BD FACS Calibur Flow Cytometer (BD Biosciences). Data analysis was performed by the FCS Express 5 Flow Cytometry software.

#### 4.4.11. Determination of cellular uptake using microscopy analysis

The MDA-MB-231 cells were cultured, as mentioned earlier.<sup>14</sup> For microscopy analysis, cells were seeded in 6-well plates at a density of  $1.5 \times 10^5$  cells per well, each well containing a sterile 22 mm square coverslip (pre-coated with 0.1% gelatin) in the bottom and incubated overnight in complete media in a CO<sub>2</sub> incubator. Cells were then treated with Dox@4.nIR12 in the presence and absence of light for 10 minutes and further incubated for 8 hours in 1% FBS containing media. Post-treatment, cells were washed with PBS buffer and fixed with 4% formaldehyde at 37 °C for 10 minutes. Coverslips were then washed thrice with PBS and treated with 0.1% Triton X-100 for 5 minutes at 37 °C. Cells were again washed thrice with PBS buffer. To counterstain the nuclei, DAPI (5 µg/mL) was added and incubated for 5 minutes. Cells were then washed thrice with PBS. Subsequently, coverslips were taken out from the wells and mounted over a glass slide. The slides were then allowed to dry for 15 mins. Finally, the microscopic analysis was performed using a confocal laser scanning microscope (CLSM; Leica TCS SP8;  $\lambda_{\text{ex}}$  = 488 nm,  $\lambda_{\text{em}}$  = 560–630 nm for Dox and  $\lambda_{\text{ex}}$  = 405 nm,  $\lambda_{\text{em}}$  = 440–480 nm for DAPI).

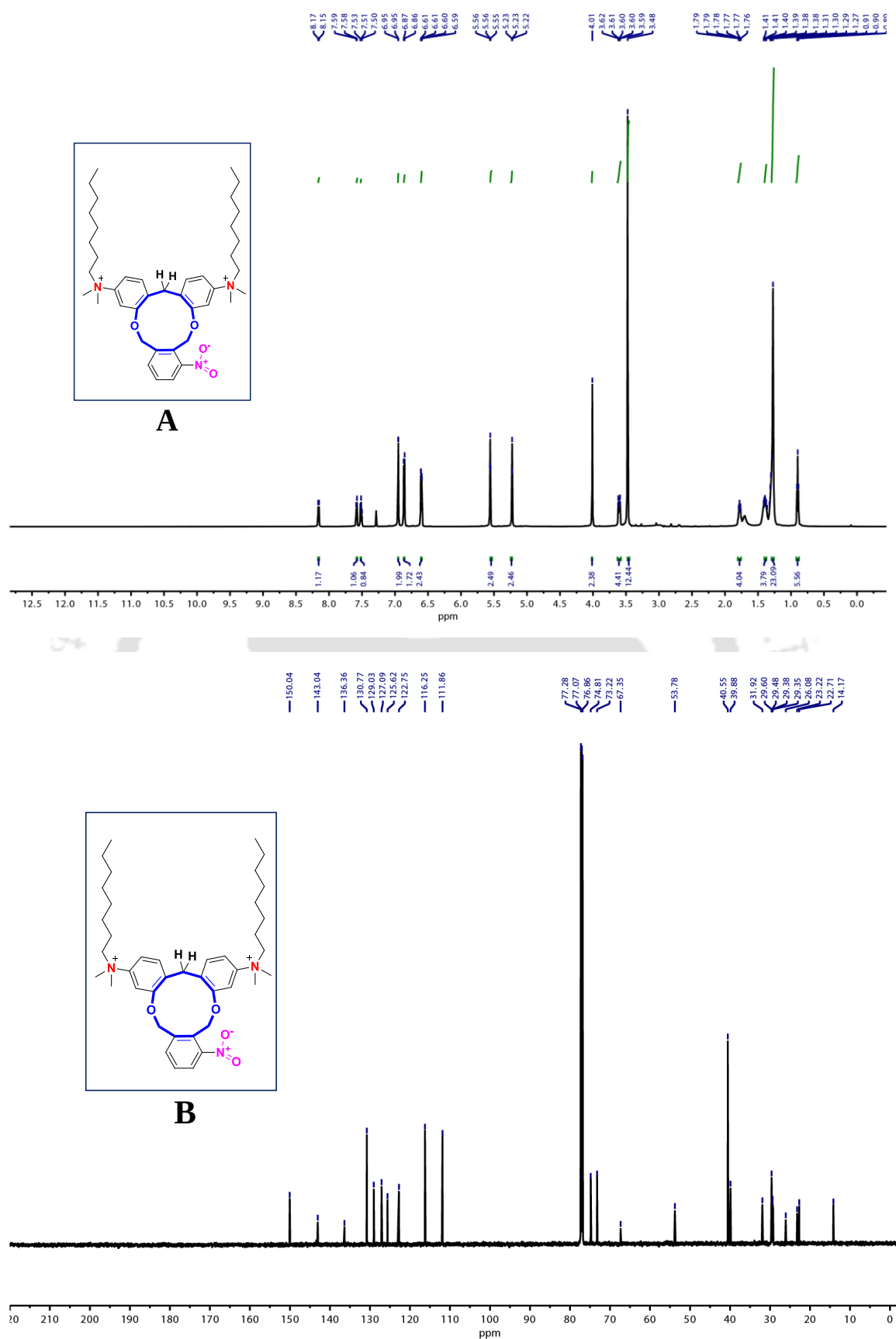
#### 4.4.12. $^1\text{H}$ NMR and $^{13}\text{C}$ NMR, HRMS spectra and HPLC traces of the synthesized compounds

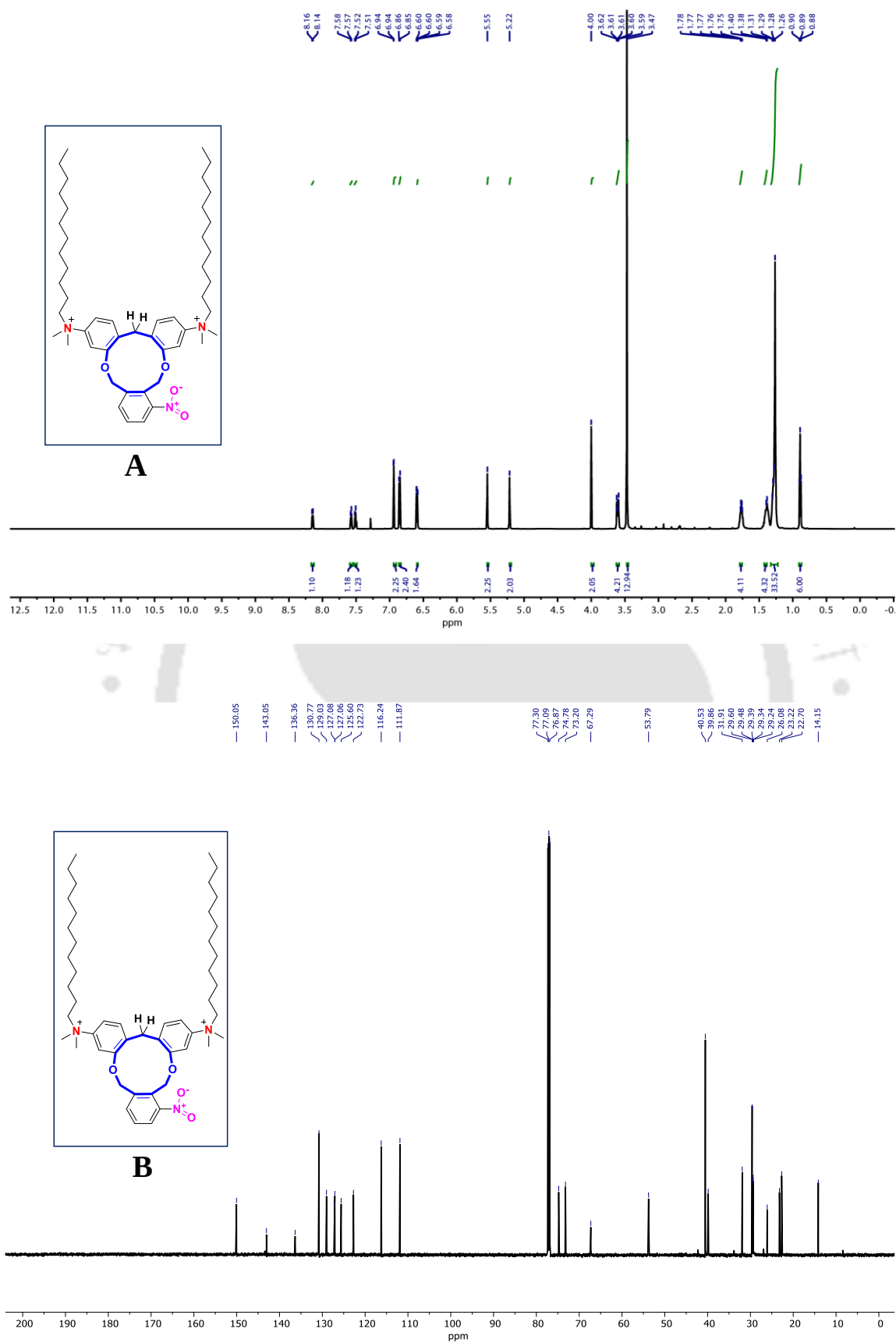


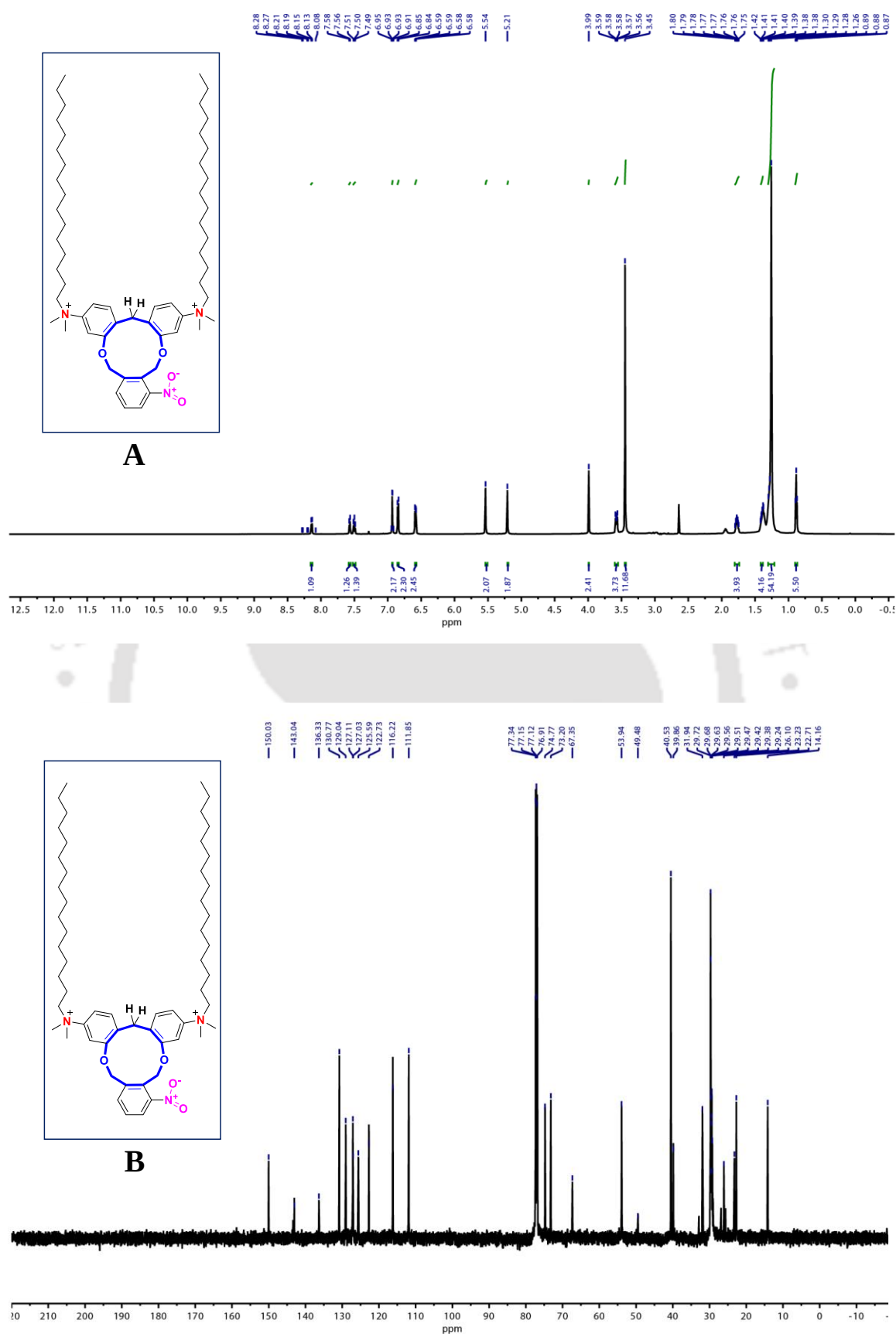
**Figure 4.7.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 4.4.











## 4.4.13. Purity analysis of the final lipid molecules

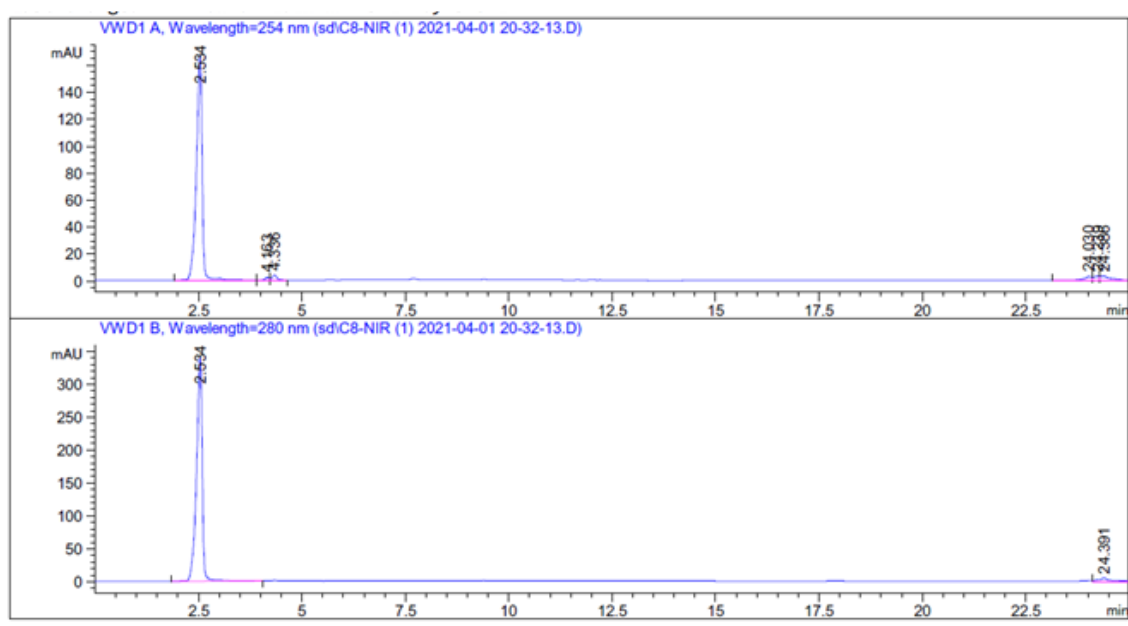


Figure 4.12. HPLC trace of compound 4.nIR8.

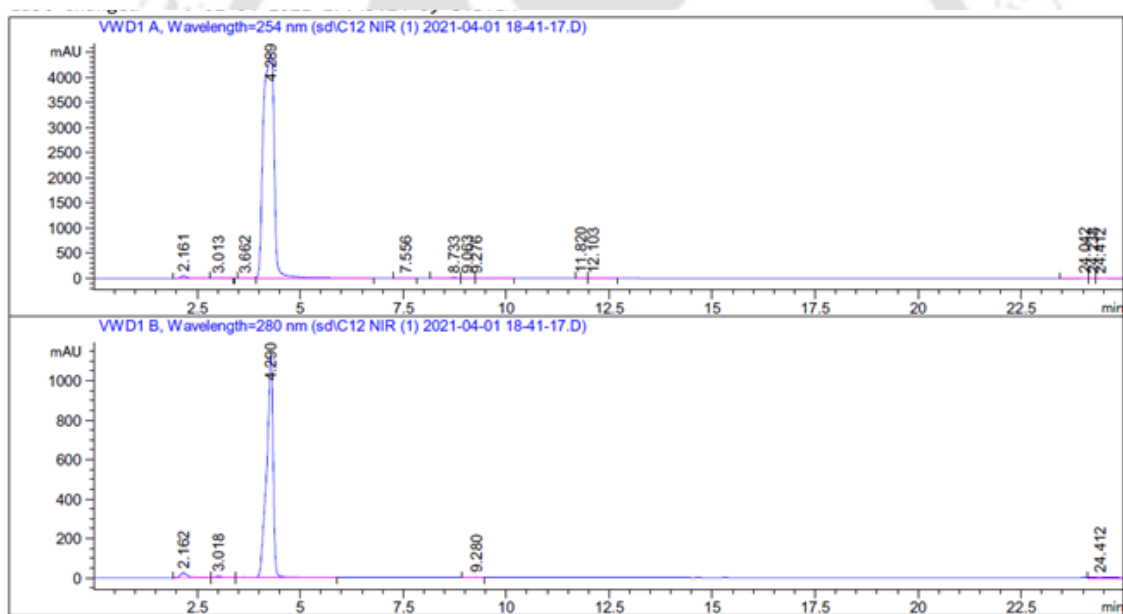


Figure 4.13. HPLC trace of compound 4.nIR12.

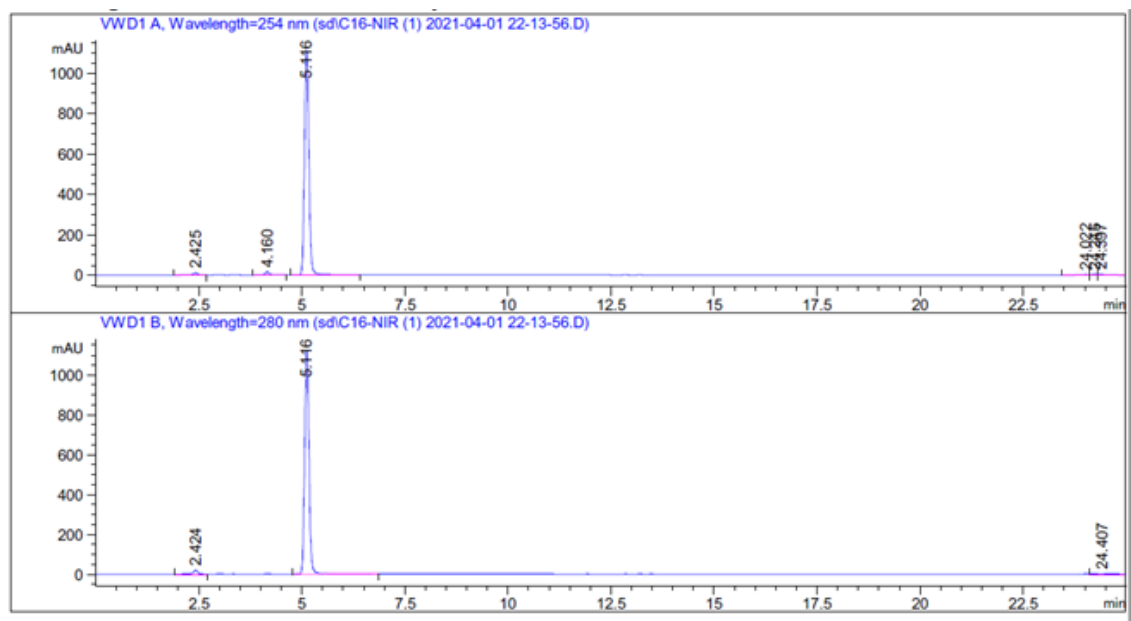


Figure 4.14. HPLC trace of compound 4.nIR16.

#### 4.4.14. Mass analysis of light mediated cleavage

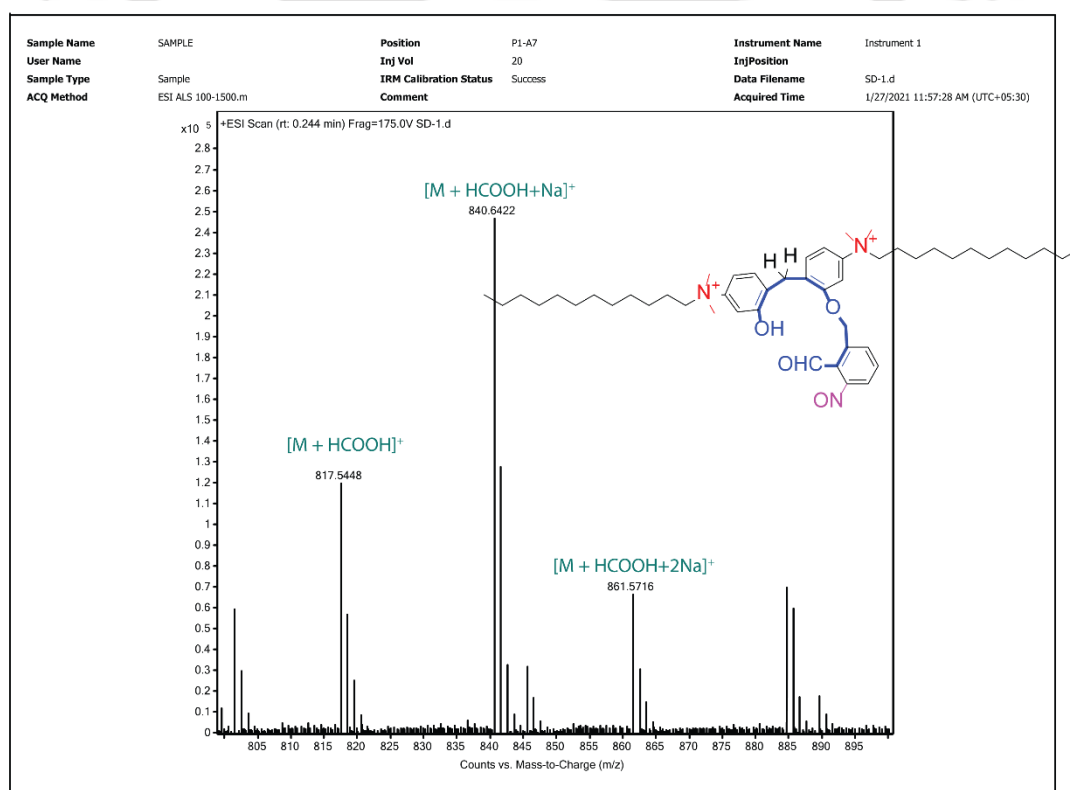
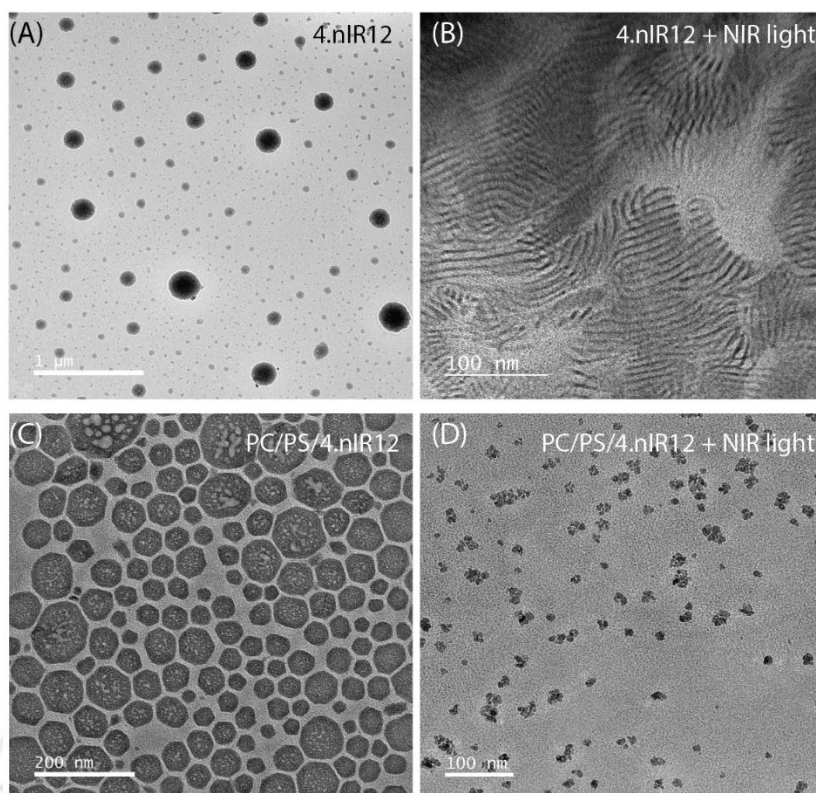


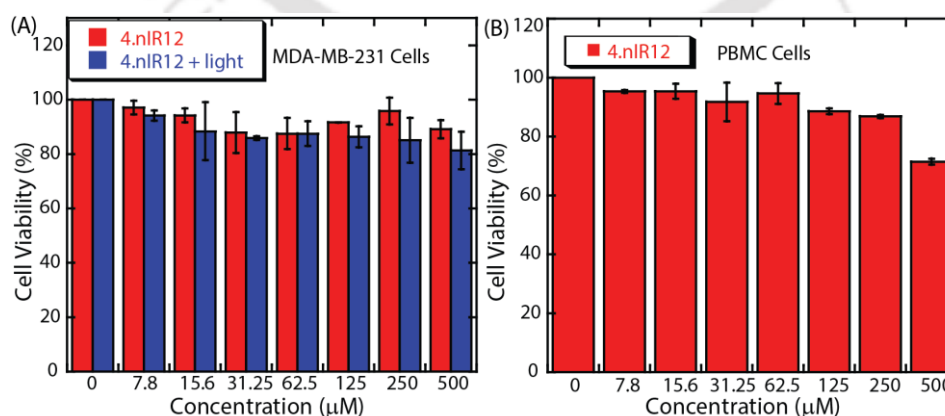
Figure 4.15. HRMS analysis of NIR light-mediated photocleavage of 4.nIR12.

#### 4.4.15. TEM images of the lipid after and before NIR light irradiation



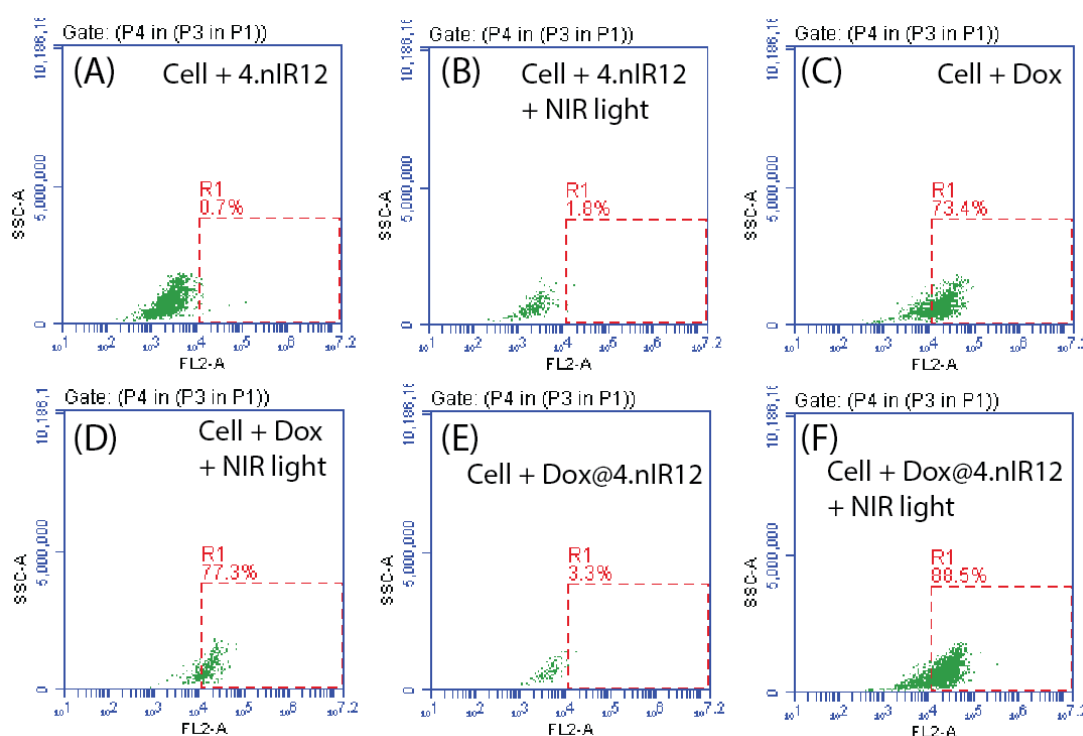
**Figure 4.16.** Representative FETEM image of the water-soluble aggregates produced from only **4.nIR12** before (A) and after (B) NIR-light treatment (808 nm, 1 W cm<sup>-2</sup>; 10 min.). Representative FETEM image of the lipid mixture produced from DPPC/DPPS/**4.nIR12** before (C) and after (D) NIR-light treatment (808 nm, 1 W cm<sup>-2</sup>; 10 min.).

#### 4.4.16. Cell viability analysis



**Figure 4.17.** Viabilities of MDA-MB-231 (A) and PBMC (B) cells in the presence of **4.nIR12** (in the absence and presence of NIR light) at different concentrations.

## 4.4.15. Flow cytometry analysis



**Figure 4.18.** Flow cytometry analysis to measure the Dox uptake efficiencies of MDA-MB-231 cells treated with free Dox (125  $\mu\text{M}$ ) and Dox@4.nIR12 (1.25  $\mu\text{M}$  of Dox and 125  $\mu\text{M}$  of amphiphile) in the absence and presence of NIR light (808 nm, 1  $\text{W cm}^{-2}$ , 10 min.). The inherent fluorescence property of Dox molecules was used for the measurement (FL2-A channel). Only cells were used as control. Cell + 4.nIR12 (A); Cell + 4.nIR12 + NIR light (B); Cell + Dox (C); (Cell + Dox) + NIR light (D); Cell + Dox@4.nIR12 (E); Cell+ Dox@4.nIR12 + NIR light (F).

## 4.5. References

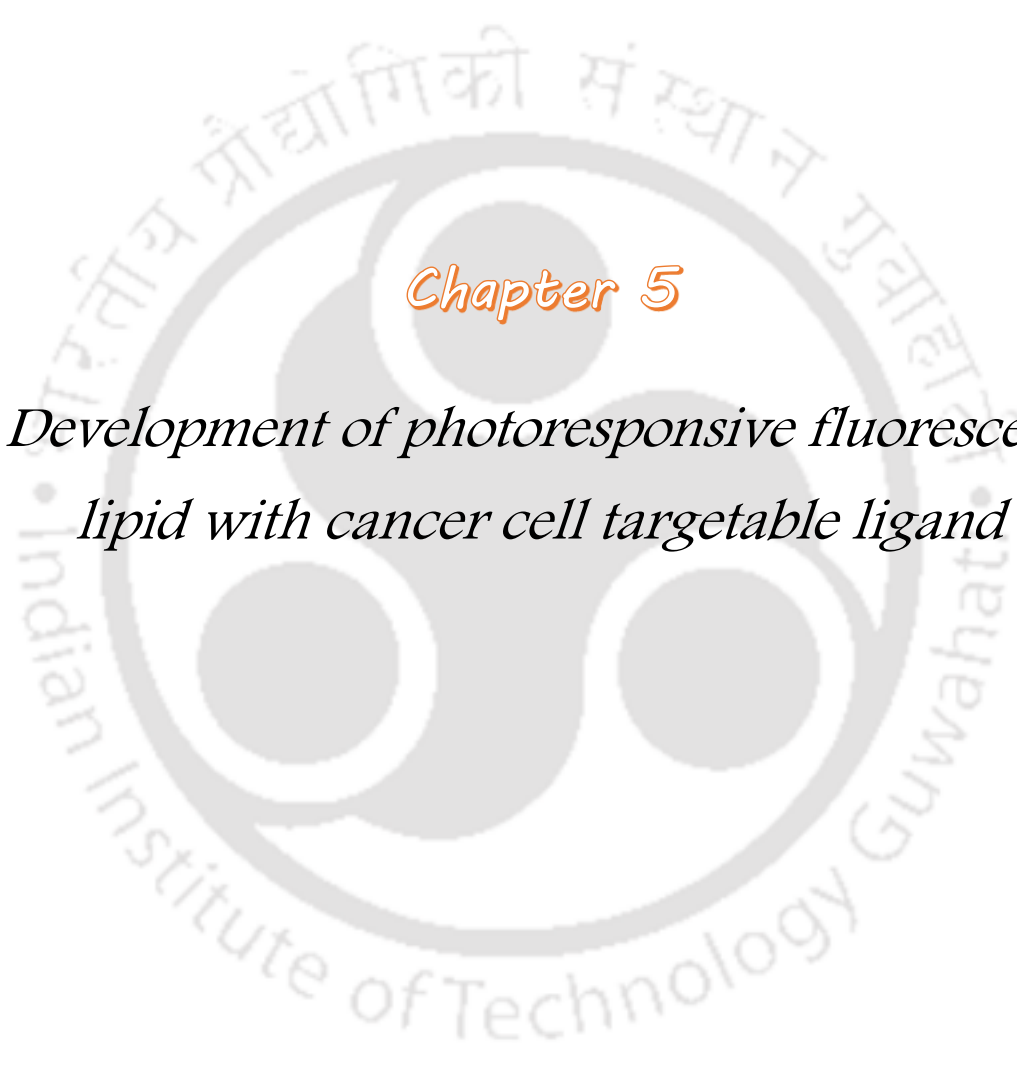
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The background of the slide features a large, faint watermark of the Indian Institute of Technology Guwahat logo. The logo is circular, containing a stylized 'IIT' monogram in the center. The text 'Indian Institute of Technology Guwahat' is written in English around the bottom half of the circle, and its Assamese equivalent 'গুৱাহাটীৰ ভাৰতীয় প্ৰযুক্তিবিদ্যাৰ সংস্থান' is written along the top half.

## Chapter 5

*Development of photoresponsive fluorescent lipid with cancer cell targetable ligand*





### 5.1. Background and objective of present work

The previous chapter demonstrated how NIR light irradiation causes the light responsive macrocyclic amphiphile to deliver the chemotherapeutic drug doxorubicine to specific sites. Drug delivery systems (DDSs) can significantly reduce the adverse side effects and improve the therapeutic efficacy of the chemotherapeutic agents. In addition, early diagnosis and image-guided chemotherapy can substantially reduce the mortality rate.<sup>1a, 1b</sup> The delivery of appropriate drug doses at the right time at the disease site is challenging. Hence, attempts are being made to convert the liposome from only DDSs into multifunctional theranostic nanoplatforms. In this regard, lysophosphatidylcholine-based porphyrinsome, BODIPYsome, and other fluorescent lipid-based molecules were developed for image-guided drug delivery applications.<sup>2</sup> In addition, various cocktail mixtures of DDSs, drug molecules, and photo-responsive molecular probes were recently demonstrated for chemotherapeutic treatments.<sup>3</sup> However, pre-clinical and clinical validation of each molecule associated with this cocktail mixture increase the time and economic burden of the drug discovery process. The UV-light mediated drug release properties also limit their application.<sup>4</sup> Lack of targetability complicates further applications of these DDSs in theranostics.<sup>5</sup> Thus, a major goal in chemotherapeutic DDS-related research is to develop a new strategy that could be used to identify and irradiate the malignant tumor cells effectively.

The malignant tumor cells upregulate the expression of various tumor-associated molecules, such as lactate dehydrogenase (LDH), carbonic anhydrase-IX (CA-IX), due to the activity of hypoxia-inducible factors (HIF-1 $\alpha$ ).<sup>6</sup> Recent studies revealed that these surface-exposed proteins could be utilized to selectively deliver the chemotherapeutic drugs molecules to the cancer cells over the healthy cells.<sup>7-9</sup> In particular, the overexpression of CA-IX is directly associated with the multidrug resistance and aggressiveness of the tumor cells. Consequently, inhibition of CA-IX or targeting CA-IX to deliver drug molecules to the cancer cells is considered to have fewer side effects and higher therapeutic efficacy.<sup>4, 6, 10</sup> Recently, various CA-IX targetting and endogeneous stimuli-responsive DDSs were developed.<sup>11, 12</sup> However, we cannot completely rely on endogenous stimuli (such as pH, redox potential, hypoxic environment, protein/enzyme levels) because they sometimes show insignificant differences between normal and cancer cells, resulting in unwanted



selectivity and off-target drug release. They mostly rely on the differences in properties between cancerous and normal cells, which primarily depends on the pathogenic conditions.<sup>13</sup> Exogenous stimuli (laser light, magnetic field, ultrasound) are considered more beneficial for the precise delivery of drug molecules.<sup>14, 15</sup> The use of near-infrared (NIR) light provides multiple advantages, including negligible phototoxicity, higher biological tissue penetration ability, fast and superior spatiotemporal drug release efficacy.<sup>15</sup>

Early diagnosis or image-guided chemotherapy is also challenging due to the lack of suitable early diagnostic technology and the severe side effects associated with the nontargeted fluorophore resulting from a lack of specificity for cancer cells. Thus, the development of targeted imaging agents is highly beneficial for diagnosis.<sup>16-18</sup> The NIR-light-responsiveness could provide on-demand delivery of appropriate drug doses in a spatially- and temporally-controlled manner for surface and deep-tissue cancer treatment. Therefore, developing multifunctional DDSs with fluorescence properties, targetability, and stimuli-responsive spatiotemporally controllable drug-release profiles is of great interest for chemotherapy.

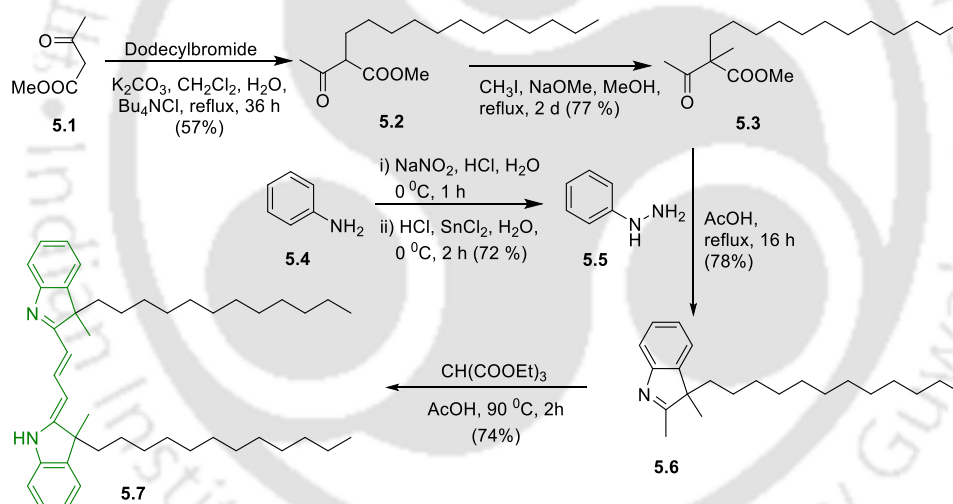
Our previously developed NIR-light-responsive macrocyclic gemini amphiphiles that produce spherical aggregates in the aqueous environment. The photo-induced cleavage of the strained dioxacycloundecine ring promotes the formation of the nanotubular assembly, which causes the release of encapsulated drug molecules to the cancer cells in a controlled manner.<sup>19</sup> Inspired by the successful drug delivery application of the NIR-light-responsive macrocyclic amphiphiles, we developed acetazolamide-grafted cyanine-3 (Cy3) dye-based NIR-responsive macrocyclic amphiphiles. The Cy3 dye is useful for both fluorescence and scattering-based imaging.<sup>20</sup> The acetazolamide moiety present on the outer surface of the liposome could bind to the tumor-specific CA-IX receptor and deliver the chemotherapeutic drug molecules to the cancer cells.<sup>4</sup> The presence of a strained 12-membered cyclic ring and a photon absorbing o-nitrobenzyl moiety would make the amphiphile NIR-light-responsive.<sup>19</sup> In addition, the presence of Cy3 dye in the core group of the amphiphile could be useful to monitor the cellular uptake and localization of the drug delivery systems. The synthesized Cy-3-based fluorescent amphiphile exhibits a visible J-aggregation bathochromic shift in the aqueous and targeted drug delivery efficacy to the cancer cells. Photo-induced irreversible

transformation spherical to nanotubular self-assembly and the presence of cancer cell recognizing ligand is appealing for various applications, including targeted drug delivery and early diagnosis or image-guided chemotherapy.

## 5.2. Results and discussions

### 5.2.1. Synthesis of photoresponsive fluorescent lipids

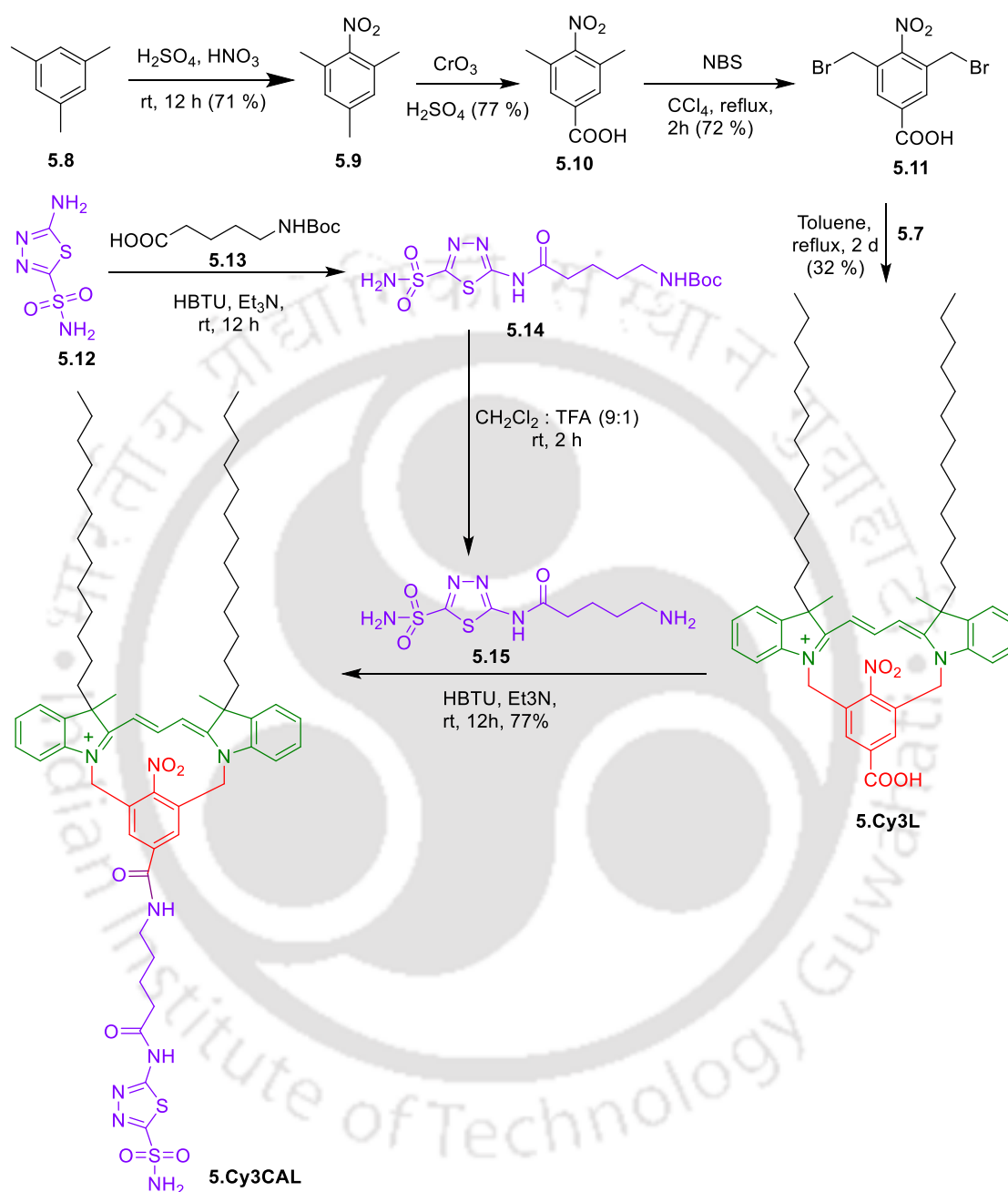
The modular amphiphile was synthesized in a stepwise manner (Figures 5.1 and 5.2). Controlled di-alkylation of methyl 3-oxobutanoate provided the methyl 2-acetyl-2-methyltetradecanoate (**5.3**). Then 3-dodecyl-2,3-dimethyl-3*H*-indole (**5.6**) was synthesized from compound **5.3** and phenylhydrazine (**5.5**) following the Fischer-indole synthesis procedure. Condensation of compound **6** with triethyl orthoformate provided the Cy3 derivative, 3-dodecyl-2-((*E*)-3-((*E*)-3-dodecyl-3-methylindolin-2-ylidene)prop-1-en-1-yl)-3-methyl-3*H*-indole (**5.7**).



**Scheme 5.1.** Synthesis of 3-dodecyl-2-((*E*)-3-((*E*)-3-dodecyl-3-methylindolin-2-ylidene)prop-1-en-1-yl)-3-methyl-3*H*-indole (**5.7**).

Subsequent ring-closing reaction *via* nucleophilic substitution between compound **5.7** and 3,5-bis(bromomethyl)-4-nitrobenzoic acid (**5.11**) resulted the 12-member macrocyclic compound (5<sup>2*Z*,7*E*</sup>)-3<sup>5</sup>-carboxy-1<sup>3</sup>,5<sup>3</sup>-didodecyl-1<sup>3</sup>,5<sup>3</sup>-dimethyl-3<sup>2</sup>-nitro-1<sup>3*H*</sup>-1,5(1,2)-diindol-1-iuma-3(1,3)-benzenacyclooctaphan-7-en-1<sup>1</sup>-ium (**5.Cy3L**). Finally, the acid amine coupling reaction between **5.Cy3L** and 5-amino-*N*-

(5-sulfamoyl-1,3,4-thiadiazol-2-yl)pentanamide (**5.15**) yielded the targeted **5.Cy3CAL** amphiphile.

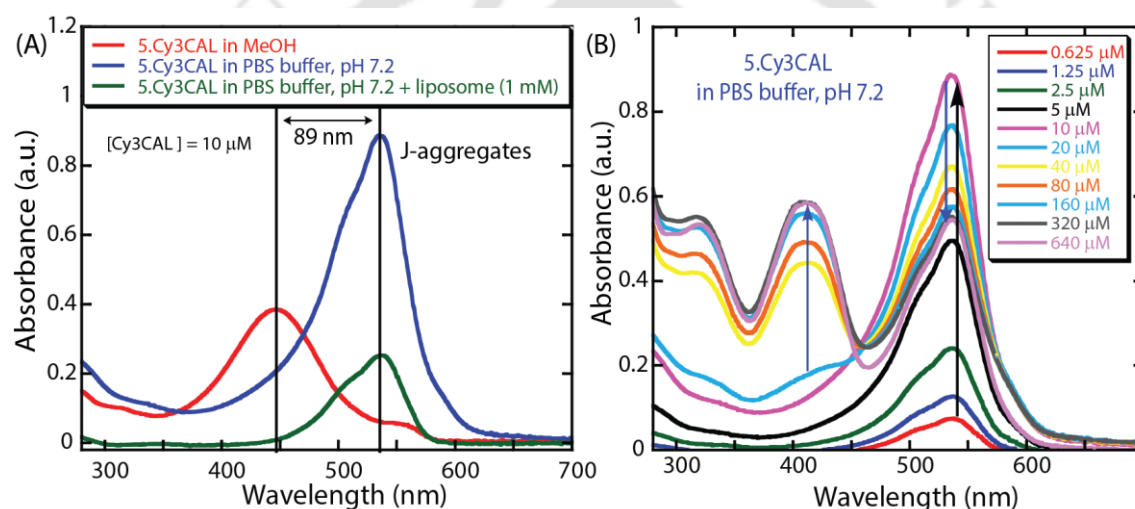


**Scheme 5.1.** Synthesis of the targeted lipid **5.Cy3CAL**.

The synthesized photo-responsive fluorescent macrocyclic **5.Cy3CAL** amphiphile is structurally different from the naturally occurring phospholipids. Hence, the biophysical properties, biocompatibility, and drug delivery efficacy of the amphiphile were systematically investigated.

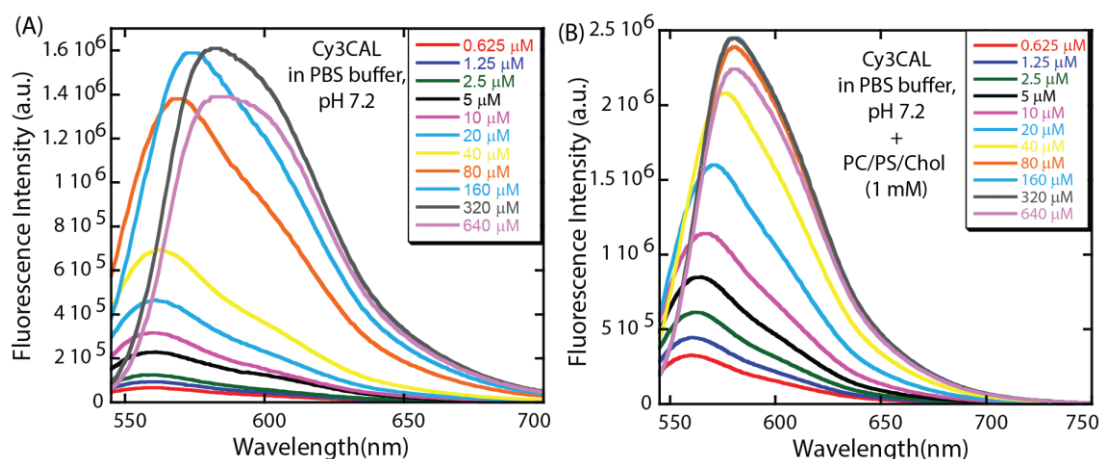
### 5.2.2. Spectroscopic studies of the lipid 5.Cy3CAL

The presence of cyanine moiety within the core of the targeted amphiphiles prompted us to investigate their spectral properties. The absorption spectra of **5.Cy3CAL** displayed a bathochromic shift ( $\lambda_{\text{ex}} = 535 \text{ nm}$ ) in the aqueous medium (PBS buffer pH 7.2) compared to that in the methanol medium (monomer;  $\lambda_{\text{ex}} = 448 \text{ nm}$ ), suggesting the formation of J-aggregates (Figure 5.1A). However, at higher concentrations, the absorption spectra displayed a hypsochromic shift in the aqueous medium, suggesting the formation of an H-like tetramer aggregation pattern or coexistence of J-aggregates with a minority population of disordered aggregates (Figure 5.1B).<sup>21, 22</sup>



**Figure 5.1.** UV-Vis spectra of **5.Cy3CAL** (10 μM) in MeOH, PBS buffer, pH 7.2 and in the presence of liposomes of PC/PS/Chol (60:20:20) (A). Concentration-dependent UV-Vis spectra of **5.Cy3CAL** in PBS buffer, pH 7.2 (B).

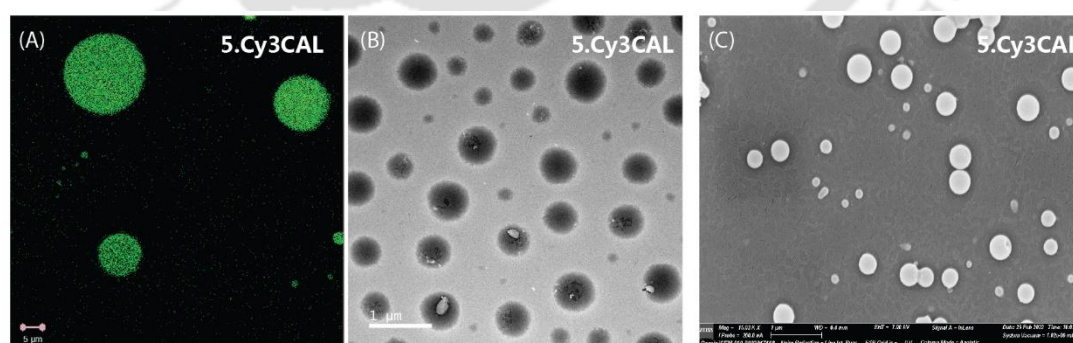
The fluorescence spectral analysis ( $\lambda_{\text{ex}} = 535 \text{ nm}$  and  $\lambda_{\text{em}} = 560 \text{ nm}$ ) revealed that the critical aggregation constants (CACs) of **5.Cy3CAL** are around 6.67 μM (CAC1) and 108.89 μM (CAC2) in PBS buffer pH 7.2 (Figure 5.24). Meanwhile, the **5.Cy3CAL** showed similar fluorescence spectral properties but with higher intensities in the presence of model liposomes (PC/PS/Chol in the mol ratio of 60:20:20) (Figure 5.2 and 5.7B). The increase in the fluorescence intensity could be due to the localization of **5.Cy3CAL** within the hydrophobic environment of the lipid bilayer.



**Figure 5.2.** Concentration-dependent fluorescence spectra of **5.Cy3CAL** in PBS buffer, pH 7.2 (A) and in the presence of liposomes of PC/PS/Chol (60:20:20) (B).

### 5.2.3. Morphological analysis of the vesicle of lipid **5.Cy3CAL**

Due to the amphiphilic nature, the **5.Cy3CAL** is anticipated to form soluble aggregates in the aqueous medium. The confocal laser scanning microscopic (CLSM) analysis of the giant unilamellar vesicles (GUVs) of only **5.Cy3CAL** demonstrates its spherical assembly in PBS buffer, pH 7.2 (Figure 5.3A), which could be due to the formation of cyclic coulomb-coupled J-aggregates.<sup>22</sup> The analysis of transmission electron microscopic (TEM) and field emission scanning electron microscopic (FESEM) images also revealed the formation of spherical NAs with diameters of 100–300 nm (Figure 5.3B and 5.3C).

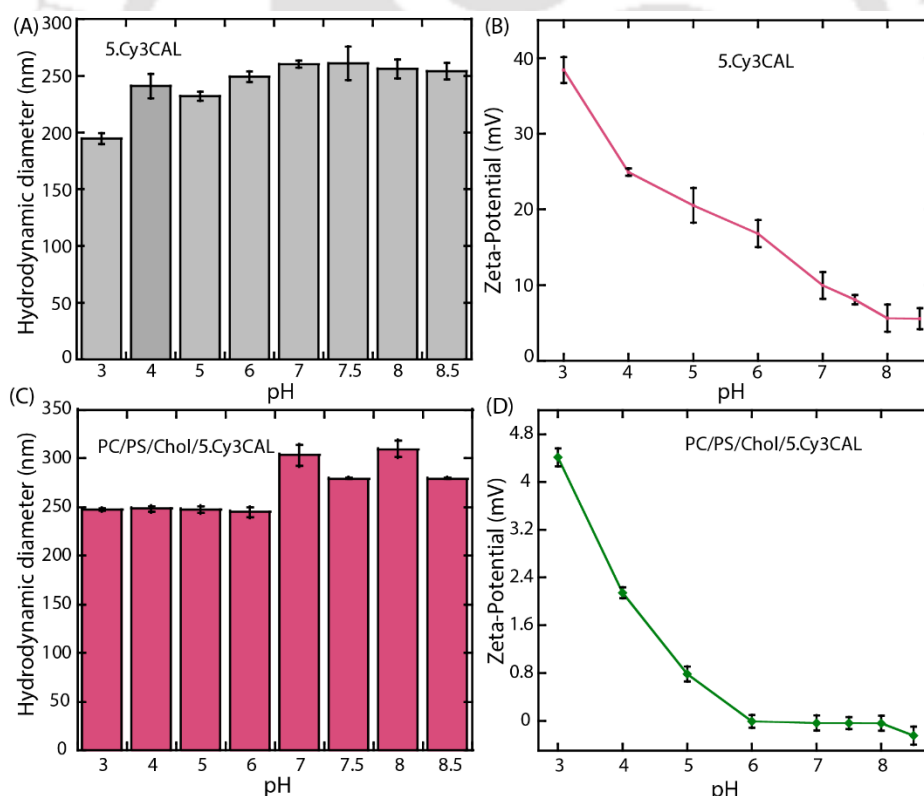


**Figure 5.3.** Representative GUV (A), TEM (B) and FESEM (C) images of **5.Cy3CAL** NAs.



### 5.2.4. Hydrodynamic diameter and zeta potential measurement of the lipid nanoaggregates

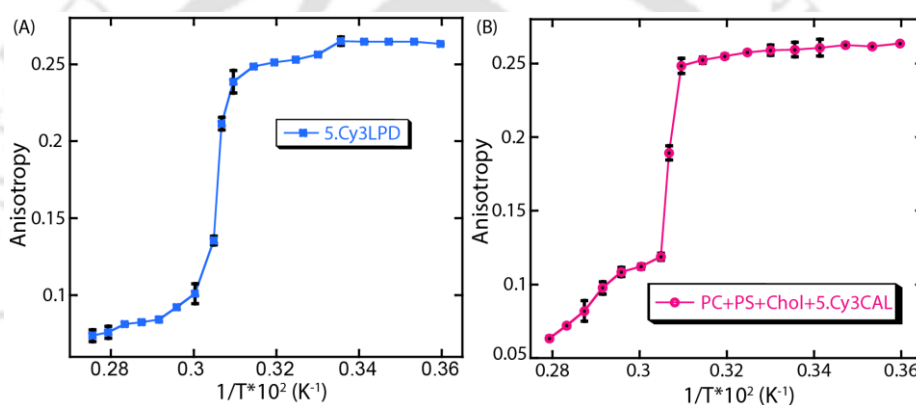
The dynamic light scattering (DLS) analysis showed that the hydrodynamic diameter ( $d_H$ ) of the vesicles varies within 225–275 nm across different pH, which signifies that the pH of the solution has a negligible effect on the hydrodynamic diameter of the vesicles (Figure 5.4A). The surface potential of the NAs was around +10.4 mV at physiological pH; however, a very high positive surface charge ( $\sim 37$  mV) was observed at pH 3.0, indicating that at lower pH, free amine at the head group gets protonated (Figure 5.4B). The surface potential decreases with the increase in pH, which minimizes the positive charge of **5.Cy3CAL** by the increasing solvation number of the negatively charged hydroxyl group. The pH-dependent surface potential of amphiphile **Cy3L** showed -5.0 mV at physiological pH due to the presence of the acid group, which acts as a head group of NAs.



**Figure 5.4.** Variation of hydrodynamic diameter (A) and zeta potential (B) of the soluble aggregates of **5.Cy3CAL** at various pH. Variation of hydrodynamic diameter (C) and zeta potential (D) of PC/PS/Chol/**5.Cy3CAL** (55/20/20/5) at various pH.

### 5.2.5. Phase transition temperature measurements

Fluorescence anisotropy measurements were performed to investigate the thermal stability of NAs of **5.Cy3CAL** in the aqueous media. The higher phase transition temperature ( $T_m = 53\text{ }^{\circ}\text{C}$ ) of the amphiphile suggests the higher thermal stability of the vesicles, which could be due to the formation of stable J-aggregates. The  $T_m$  value of the lipid mixture dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylcholine (DPPS), cholesterol (Chol), and **5.Cy3CAL** (molar ratio 55:20:20:5) was also  $53\text{ }^{\circ}\text{C}$  (Figure 5.5). The higher  $T_m$  values of only **5.Cy3CAL** and lipid mixture containing **5.Cy3CAL** demonstrate its higher thermal stability and drug retention capability, which could be useful in drug delivery and others.



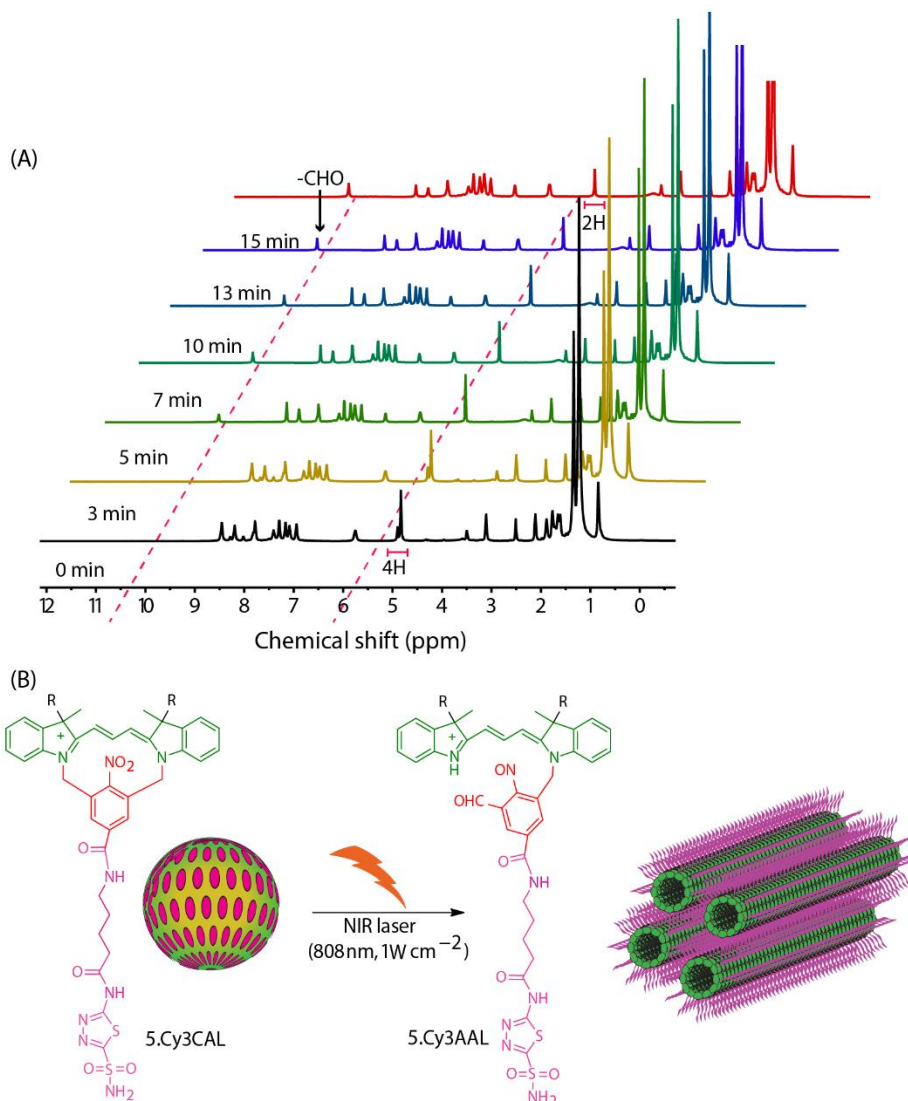
**Figure 5.5.** The temperature-dependent fluorescence anisotropy measurement of environment-sensitive 1,6-diphenyl-1,3,5-hexatriene dye incorporated within the water-soluble aggregates. The phase transition temperature of lipid **5.Cy3CAL** (A), DPPC/DPPS/Chol/**5.Cy3CAL**(55:20:20:5) (B).

### 5.2.6. NIR light-induced photocleavage of the compound

The photo-induced transformation of macrocycle to acyclic amphiphile was investigated by  $^1\text{H}$  NMR analyses. A solution of **5.Cy3CAL** in  $\text{DMSO-}d_6$  solvent was irradiated with NIR laser light ( $808\text{ nm}$ ,  $1\text{ W cm}^{-2}$ ) for 0-15 minutes. The time-dependent  $^1\text{H}$  NMR analyses displayed the disappearance of one of the benzylic protons  $\text{H}_a$  ( $\delta = 3.8\text{ ppm}$ ) after 15 minutes of light irradiation and simultaneous generation of aldehydic proton at  $\delta = 9.8\text{ ppm}$  (Figure 5.6A). The disappearance of the proton signal of  $\text{H}_a$  indicates the cleavage of the *o*-nitrobenzyl moiety and the opening of the 12-membered strained macrocyclic ring. This facile cleavage of the



macrocyclic ring by the NIR light could be due to the presence of high molecular angular strains within the Cy3-containing macrocyclic ring of the amphiphile. Hence, the presence of a photon-absorbing 2-nitrobenzyl group makes **5.Cy3CAL** amphiphile a NIR-sensitive molecule leading to the cleavage of the benzyl bond via the Norrish type II mechanism.<sup>23</sup>

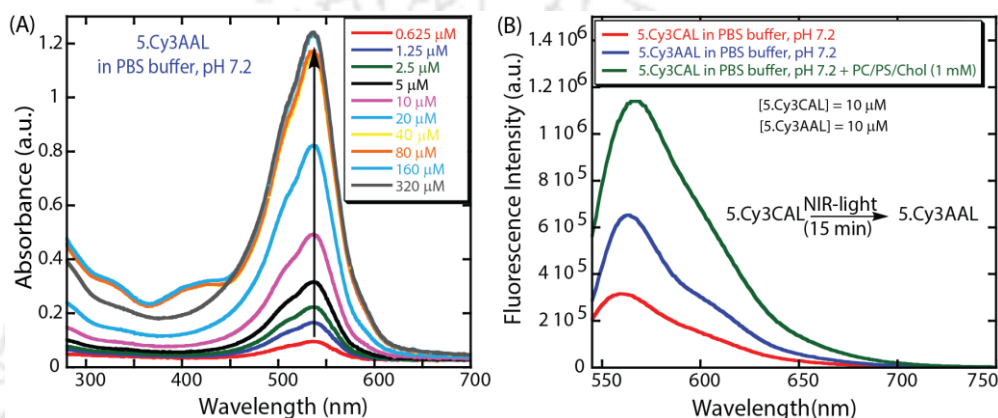


**Figure 5.6.**  $^1\text{H}$  NMR spectra of **5.Cy3CAL** without and with NIR-light treatment at different time intervals (A). Photo-induced structural changes of **5.Cy3CAL** to **5.Cy3AAL** (B).

## 5.2.7. Physical properties of NAs after light-mediated cleavage

### 5.2.7.1. Spectral properties analysis

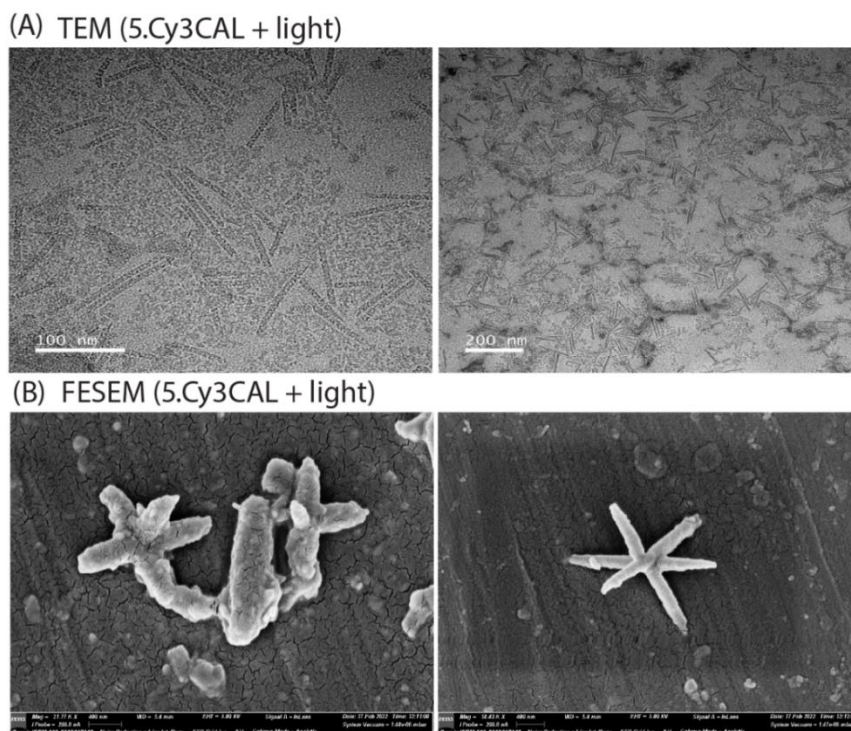
Meanwhile, the absorption spectra of **5.Cy3AAL** also displayed a bathochromic shift in the aqueous medium ( $\lambda_{\text{ex}} = 538 \text{ nm}$ ; in PBS buffer pH 7.2) compared to that in the methanol medium (monomer;  $\lambda_{\text{ex}} = 446 \text{ nm}$ ), suggesting the formation of fluorescent J-aggregates in the aqueous medium (Figure 5.7). The fluorescence spectral analysis ( $\lambda_{\text{ex}} = 538 \text{ nm}$  and  $\lambda_{\text{em}} = 565 \text{ nm}$ ) revealed that the CAC values of **5.Cy3AAL** are around 25.28 (CAC1) and 89.44 (CAC2)  $\mu\text{M}$  in PBS buffer pH 7.2 (Figure 5.24).



**Figure 5.7.** Concentration-dependent UV-Vis spectra of **5.Cy3AAL** in PBS buffer, pH 7.2 (A). Fluorescence spectra of **5.Cy3CAL** and **5.Cy3AAL** (10  $\mu\text{M}$ ) in PBS buffer, pH 7.2 and in the presence of liposomes of PC/PS/Chol (60:20:20) (A)

### 5.2.7.2. Morphology analysis

The aggregation pattern of the NAs of **5.Cy3CAL** amphiphile after light irradiation in the aqueous environment was also analyzed by the TEM and FESEM analyses. The images revealed that the spherical NAs turn into rod-like nano assembly after NIR-light treatment (Figure 5.8). The result indicates that after NIR light irradiation, the unsymmetrical (headgroup) **5.Cy3AAL** molecules could form oblique-type J-aggregation patterns to form nanotubes.<sup>24-26</sup> The formation of such fluorescent nanotubes could be useful for light-harvesting applications.<sup>27-29</sup> We hypothesized that this photo-induced conformational change could release the encapsulated drug molecules for the NAs of **5.Cy3CAL** amphiphile. The  $^1\text{H}$  NMR analysis also revealed that the Cy3 moiety of compounds **5.7**, **5.Cy3CAL**, and **5.Cy3AAL** remained in trans-conformation.

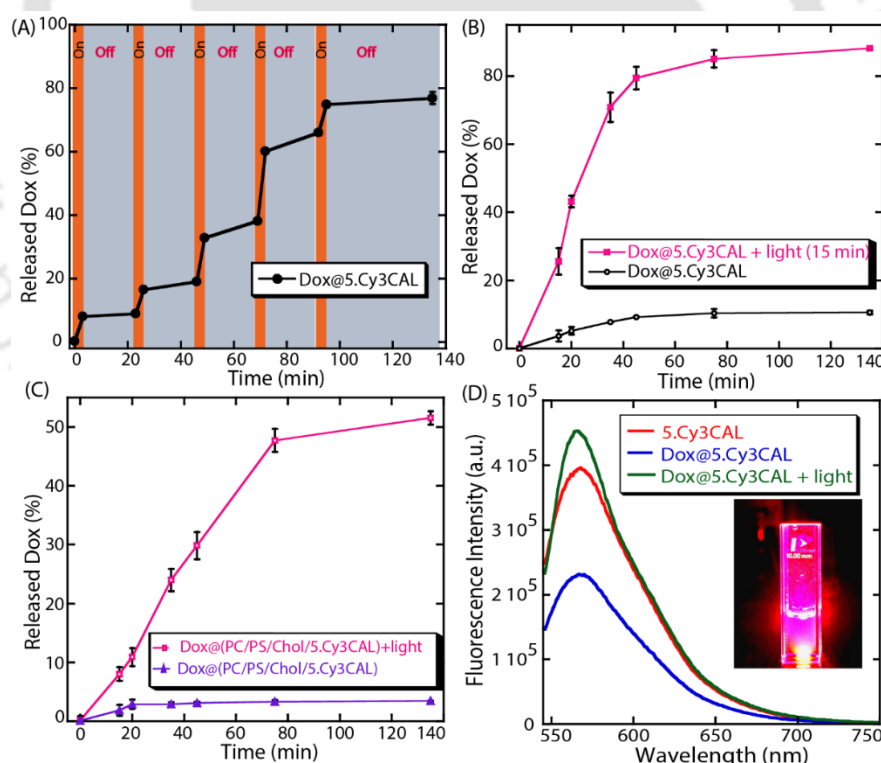


**Figure 5.8.** Representative TEM images of **5.Cy3CAL** after light treatment (A). Representative FESEM images of **5.Cy3CAL** after light treatment (B).

### 5.2.8. Drug loading and release efficiency of vesicle of 5.Cy3CAL lipid

This NIR-responsive transformation from spherical to nanotubular self-assembly pattern of **5.Cy3CAL** prompted us to investigate its drug encapsulation and release profiles. The **5.Cy3CAL** amphiphile alone and DPPC/DPPS/Chol/**5.Cy3CAL** lipid mixture (55:20:20:5) showed moderate loading capacity of the hydrophobic Dox molecules (51 and 57%, respectively at pH 7.4), indicating the successful entrapment of the Dox molecules within the hydrophobic core of the NAs. The HPLC-based Dox release profile of **5.Cy3CAL** amphiphile without and with photo-irradiation (15 min) revealed 10% and 88% release efficacies after 2 h (Figure 5.9B). The fast release profile after photo-irradiation suggests that the conformational changes allow the release of encapsulated Dox molecule, whereas, in the absence of light irradiation, no significant changes occur within the NAs. This faster drug release profile is beneficial for spatiotemporally controllable drug delivery applications, reducing the side effect due to the nonspecific delivery to the healthy cells and increasing the local concentration of the drug molecules within the cancer cells/tissues. The lipid mixture showed 4% and 51% Dox release efficiency (after 2h)

without and with photo-irradiation (15 min), respectively (Figure 5.9C). A significant reduction of Dox release efficiency suggests that the drug release profile can be tuned by altering the lipid composition. To investigate the remote and precise drug release efficiency of **5.Cy3CAL**, the liposome-containing buffer solution was irradiated with NIR light for on-off cycles (Figure 5.9A) demonstrated the pulsed Dox release profile of **5.Cy3CAL**. Because the NIR-light can be operated in a spatially- and temporally-controlled manner for surface and deep-tissue cancer treatment, we hypothesize that the **5.Cy3CAL**-based drug delivery system is quite promising to deliver and release the chemotherapeutic agents in a controlled fashion. Meanwhile, the Dox-loaded **5.Cy3CAL** showed quenching of fluorescence signal, and light-treated **5.Cy3CAL** showed enhancement in fluorescence signals (Figure 5.9D). The quenching of the **5.Cy3CAL** fluorescence signal could be due to the transfer of energy from Cy3 to Dox. The enhancement of **5.Cy3CAL** fluorescence intensity after light treatment could be useful for in situ monitoring of drug release properties.



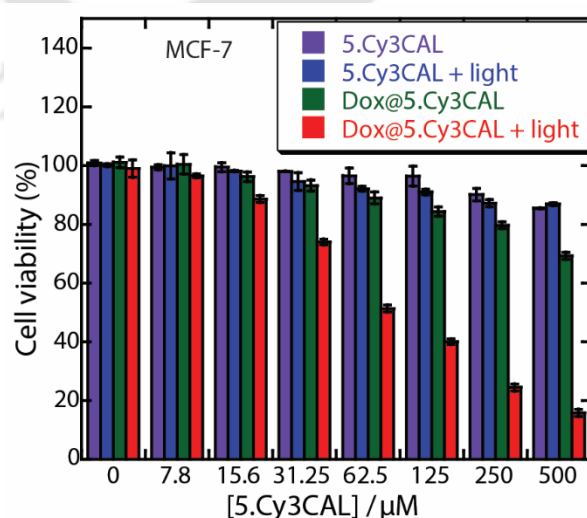
**Figure 5.9.** To attain the required doses, the extent of Dox release can be stimulated by NIR light multiple times (A). The Dox release profiles of **5.Cy3CAL** without and with NIR-light treatment (15 min) (B). The Dox release profiles of lipid mixture of PC/PS/Chol/**5.Cy3CAL** without and with NIR-light treatment (15 min) (C). NIR laser

light (808 nm, 1 W cm<sup>-2</sup>) source was used irradiation of the samples for 15 minutes. Fluorescence properties of **5.Cy3CAL**, Dox@**5.Cy3CAL**, and Dox@**5.Cy3CAL** after light treatments (D).

## 5.2.9. Cell viability and cellular uptake analysis

### 5.2.9.1. Cell Viability

The viability analysis of **5.Cy3CAL** amphiphile in MCF-7 (human breast cancer), MDA-MB468 (human breast cancer), and HEK293 (human embryonic kidney) cell lines revealed a low or insignificant toxic effect (after 48 h) without or with NIR-light treatment, demonstrating excellent biocompatibility of **5.Cy3CAL** for drug delivery applications. The CA-IX is known to be overexpressed in MCF-7 and MDA-MB-468 cell lines.<sup>30, 31</sup> Whereas, the HEK293 is a CA-IX negative cell line. The MTT analysis revealed that MCF-7 cells were viable in the presence of control amphiphile **5.Cy3L** (without or with NIR-light treatment). The MTT-based cell viability analysis revealed that the IC<sub>50</sub> values of Dox alone in MCF-7 cell lines was  $0.89 \pm 0.26$   $\mu$ M. In contrast, Dox encapsulated **5.Cy3CAL** (Dox@ **5.Cy3CAL**) showed a significant reduction of cell viability (IC<sub>50</sub> =  $0.39 \pm 0.02$   $\mu$ M with respect to Dox concentration) or  $59.70 \pm 1.20$   $\mu$ M (with respect to amphiphile concentration) upon NIR-light irradiation for 15 min in comparison to that without irradiation (Figure 5.10 and 5.25). The Dox encapsulated **5.Cy3L** (Dox@**5.Cy3L**) did not significantly affect the viability of MCF-7 cells.

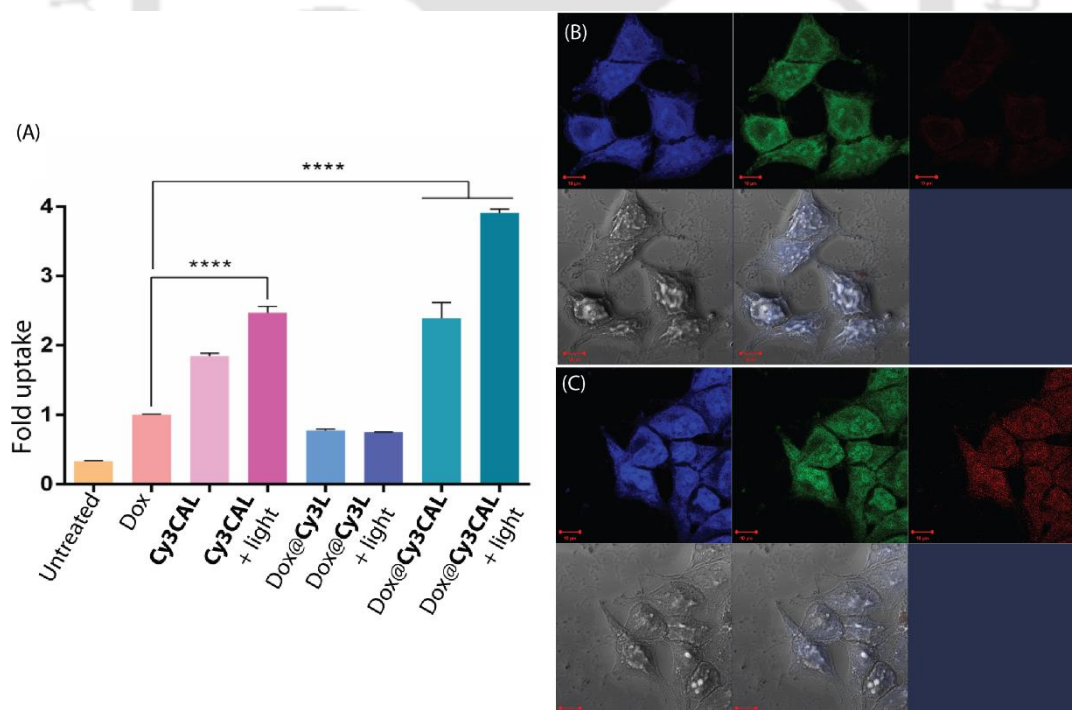


**Figure 5.10.** Viabilities of MCF-7 cells in the presence of **5.Cy3CAL**, Dox@**5.Cy3CAL** without and with NIR-light irradiation (A).



### 5.2.9.2. Cellular uptake

The CLSM and flow cytometry analysis showed the uptake of **5.Cy3CAL** to MCF-7 cells (Figure 5B-D and S8). The flow cytometry and CLSM image analyses also revealed the Dox release efficacy of **5.Cy3CAL** after NIR-light. The fluorescence (green color) of the **5.Cy3CAL** provides direct evidence for the successful internalization of **5.Cy3CAL** to the MCF-7 cells. Additionally, the flow cytometry analysis was performed to explore the apoptotic population after 48 h of treatment with **5.Cy3CAL** (Figure 5.11A). It was observed that the Dox-loaded lipid nanoparticles upon treatment with light incurred a 40.83% increase in the apoptotic cell population in comparison to the MCF7 cells. Besides, Dox treatment alone induced a 38.2% increase in the apoptotic cell population, suggesting the retention of Dox activities of the Dox@**5.Cy3CAL** delivery system. Therefore, the MTT assay flow cytometry and CLSM results suggest the effective encapsulation and CA-IX mediated delivery and photo-release of Dox molecules to the MCF-7 cells by **5.Cy3CAL** amphiphile (Figure 5.11B, C). This target-specific drug delivery and photo-induced controllable drug release efficacy could reduce the side effect of the Dox molecules.



**Figure 5.11.** FACS analysis to investigate the uptake by MCF-7 cells (A). Representative CLSM images of the MCF-7 cells treated with Dox@**5.Cy3CAL** before (B) and after (C) NIR-light treatments. The blue channel (DAPI), green channel

(5.Cy3CAL), red channel (Dox), bright-field image, and overlay of all the channels illustrate the Dox delivery efficacy to the MCF-7 cells. Scale bars: 10  $\mu$ m. The NIR-light was treated for 15 min.

### 5.3. Summary

We have developed a target-specific photo-responsive fluorescent macrocyclic amphiphile that self-assembled into spherical nanostructures in the aqueous medium via a J-aggregation pattern and demonstrated favorable properties of DDSs. The NIR-light mediated cleavage of the *o*-nitrobenzyl containing macrocyclic ring led to the formation of nanotubular assembly, releasing encapsulated Dox molecules in a controlled manner. The spherical assembly of the amphiphile showed lower cytotoxicity, and CA-IX targeted effective cellular uptake efficiency. Further, the photocleavage of the amphiphile within the cancer cells stimulates cell death with a payload of Dox molecules. Therefore, the development of target-specific NIR-responsive self-imaging macrocyclic amphiphiles as potential DDSs could be valuable in image-guided chemotherapeutic applications.

### 5.4. Experimental section

#### 5.4.1. Synthesis of the compounds

##### 5.4.1.1. General information

All reagents used were purchased from Sigma-Aldrich, TCI chemicals, and other commercial sources and used directly without further purification. The synthesized compound was purified by column chromatography using 60 – 120 mesh silica gels. The progress of the reactions was monitored by thin-layer chromatography (TLC) on silica gel 60 F254 (0.25 mm). The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR were recorded at 600 and 150 MHz with Bruker spectrometer. The chemical shifts were reported in parts per million ( $\delta$ ) using  $\text{DMSO-}d_6$ ,  $\text{CDCl}_3$  as internal solvents. The coupling constants ( $J$  values) are reported in Hertz, and chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane using residual chloroform ( $\delta = 7.28$  for  $^1\text{H}$  NMR,  $\delta = 77.23$  for  $^{13}\text{C}$  NMR) as an internal standard. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and brs (broadened singlet). High-resolution mass spectra (HRMS) were recorded at Agilent Q-TOF mass spectrometer with Z-spray source using built-in software for analysis of the recorded



data. Dipalmitoylphosphatidylcholine (DPPC), 1,2- dipalmitoylsn-glycero-3-phospho-L-serine (DPPS), were purchased from Avanti Polar Lipids (Alabaster, AL). Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers.

#### 5.4.1.2. General procedure for the synthesis of targeted compounds

**5.4.1.2.1. Synthesis of methyl 2-acetyltetradecanoate (5.2)** – A mixture of ethyl acetoacetate (7.68 mmol),  $K_2CO_3$  (15.36 mmol), 1-bromododecane (7.68 mmol),  $Bu_4NCl$  (15.36 mmol) in  $CH_2Cl_2$  (10 mL) was added in 10 mL of water, and the solution was stirred under refluxing condition for 36 h. After cooling down, the separated organic layer was washed with 5 (N) aq. HCl (10 mL). The mixture was extracted twice with dichloromethane (DCM). The combined organic layers were filtered and evaporated under reduced pressure to afford the crude mixture. The mixture was purified by column chromatography on silica gel using a gradient solvent system of DCM and acetone (10:0.7, v/v) to provide the required compound as a pale yellow oil (yield 57%). The compound was characterized by  $^1H$  and  $^{13}C$  NMR, which is in accordance with the literature report.<sup>32</sup>

**5.4.1.2.2. Synthesis of methyl 2-acetyl-2-methyltetradecanoate (5.3)** – To the stirring solution of methyl 2-acetyltetradecanoate (5.2, 1.6 mmol) in anhydrous methanol (10 mL) was added metallic sodium (1.6 mmol). The reaction mixture was then cooled to 0 °C, and methyl iodide (1.6 mmol) was slowly added dropwise with constant stirring. The reaction mixture was refluxed for 48 h. The reaction progress was monitored by TLC. After the maximum consumption of compound 5.2, the reaction mixture cooled to room temperature, and excess methanol was evaporated under reduced pressure; 30 mL of water was added, and the resulting mixture was extracted several times with DCM. The organic layers were combined, washed with 10% aqueous solution of hydrochloric acid, dried over anhydrous  $Na_2SO_4$ , and concentrated under reduced pressure to yield a crude mixture, which was purified by column chromatography on silica gel using a gradient solvent system of DCM and acetone (10:0.4, v/v) to provide the required compound as a pale yellow oil (yield 77%). The compound was characterized by  $^1H$  and  $^{13}C$  NMR, which is in accordance with the literature report.<sup>33</sup>

**5.4.1.2.3. Synthesis of phenylhydrazine (5.5)** – To the stirring solution of aniline (1g, 10.73 mmol) in EtOH (20 mL) and concentrated HCl (15 ml) was added to an ice-cold solution of sodium nitrite (740 mg, 10.73 mmol) in water (5 ml) dropwise over at the period 10 min at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. Then a solution of tin (II) chloride dihydrate (10.1 g, 53.6 mmol) in concentrated HCl (20 ml) was added dropwise over 20 minutes. The obtained precipitate was filtered and suspended in water 50 mL and then decomposed with 80 mL of 1(N) NaOH solution. The product formed was extracted with EtOAc. The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>), and the solvent was removed under reduced pressure to afford compound **5.5** as a pale yellow liquid (yield 72%). The compound was characterized by <sup>1</sup>H and <sup>13</sup>C NMR, which is in accordance with the literature report.<sup>34</sup>

**5.4.1.2.4. Synthesis of 3-dodecyl-2,3-dimethyl-3H-indole (5.6)** – To the stirring solution of phenyl hydrazine hydrochloride (**5.5**, 7.38 mmol) in acetic acid (10 mL) was added methyl 2-acetyl-2-methyltetradecanoate (**5.3**, 14.79 mmol). The reaction mixture was stirred under reflux condition for 16 h under a nitrogen atmosphere. The reaction mixture was cooled and then solvent was removed under reduced pressure. The reaction mixture was purified by column chromatography on silica gel using a gradient solvent system of hexane and ethyl acetate (10:0.5, v/v) to provide the required compound as a yellow viscous liquid (yield 78%). **Characterization of compound 5.6:** <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ<sub>ppm</sub> 7.42 – 7.38 (m, 2H), 7.26 (t, *J* = 6 Hz, 2H), 7.17 (t, *J* = 6 Hz, 2H), 3.52 (s, 3H), 2.20 (s, 2H), 1.82 – 1.75 (m, 2H), 1.38 – 1.32 (m, 2H), 1.27 – 1.24 (m, 23H), 0.85 (t, *J* = 6 Hz, 3H); <sup>13</sup>C NMR (150 MHz, DMSO-*d*<sub>6</sub>) δ<sub>ppm</sub> 188.3, 153.9, 146.3, 127.7, 125.2, 122.0, 119.6, 53.6, 39.8, 35.6, 32.6, 31.7, 29.4, 29.3, 29.1, 28.5, 27.9, 23.0, 22.5, 15.4, 14.4. HRMS (ESI) calcd. For C<sub>22</sub>H<sub>35</sub>N [M + K]<sup>+</sup>: 352.2407, found: 352.2405.

**5.4.1.2.5. Synthesis of 3-dodecyl-2-((E)-3-((E)-3-dodecyl-3-methylindolin-2-ylidene)prop-1-en-1-yl)-3-methyl-3H-indole (5.7)** – A mixture of 3-dodecyl-2,3-dimethyl-3H-indole (2 mmol) and ethylorthoformate (1 mmol) in acetic acid (5 mL) was stirred at reflux condition for 3 h. After maximum consumption of compound **5.6**, acetic acid was removed on a rotary evaporator under reduced pressure to

obtained red solid, the residue was purified by column chromatography on silica gel using a gradient solvent system of DCM and methanol (10:1, v/v) (yield 74%).

**Characterization of compound 5.7:**  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta_{\text{ppm}}$  7.90 (t,  $J = 12$  Hz, 1H), 7.37 – 7.33 (m, 2H), 7.21 – 7.18 (m, 2H), 7.11 – 7.10 (m, 2H), 6.99 – 6.97 (m, 2H), 5.81 (d,  $J = 6$  Hz, 2H), 1.91 (s, 3H) ( $-\text{CH}_3$  proton peak from counter anion  $\text{CH}_3\text{COO}^-$ ), 1.78 (p,  $J = 6$  Hz, 4H), 1.37 (s, 8H), 1.29 – 1.24 (m, 35H), 0.85 (t, 6H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{DMSO-}d_6$ )  $\delta_{\text{ppm}}$  177.6 ( $-\text{C}=\text{O}$  peak from counter anion  $\text{CH}_3\text{COO}^-$ ), 148.6, 147.3, 139.5, 133.6, 129.9, 129.0, 128.3, 123.4, 121.8, 113.2, 67.9, 49.2, 34.0, 32.8, 31.9, 29.6, 29.5, 29.4, 29.3, 28.7, 28.2, 26.1, 22.7, 14.1. HRMS (ESI) calcd. For  $\text{C}_{45}\text{H}_{68}\text{N}_2\text{K}$   $[\text{M} + \text{K}]^+$ : 659.5275, found: 659.5273.

**5.4.1.2.6. Synthesis of 1,3,5-trimethyl-2-nitrobenzene (5.9)** – To the stirring solution of mesitylene (**5.8**, 8.32 mmol) in the cold concentrated sulfuric acid (0.8 mL) was added concentrated nitric acid (0.7 mL), and the reaction mixture was stirred for 20 min. Then the resulting solution was stirred overnight at room temperature. The reaction progress was monitored by TLC. After the maximum consumption of the mesitylene, the residue was poured into cold deionized water and then was extracted with ethyl acetate. The organic layers were washed with saturated sodium bicarbonate ( $\text{NaHCO}_3$ ) water, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , then filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using hexane and ethyl acetate (1:15, v/v) as an eluent to afford compound **5.9** as light yellow solid (yield 71%). The compound was characterized by  $^1\text{H}$  and  $^{13}\text{C}$  NMR, which is in accordance with the literature report.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  6.93 (s, 2H), 2.33 (s, 3H), 2.30 (s, 6H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  149.7, 140.3, 129.5, 21.0, 17.5. ESI-MS calcd. For  $\text{C}_9\text{H}_{11}\text{NO}_2$   $[\text{M}]^+$ : 165.0790, found: 165.5253.<sup>35</sup>

**5.4.1.2.7. Synthesis of 3,5-dimethyl-4-nitrobenzoic acid (5.10)** – To the stirring solution of **5.9** (6.05 mmol) and chromium trioxide (18.09 mmol) was added to acetic acid (8 mL), the reaction mixture was refluxed overnight. The reaction was monitored by TLC. After the completion of the reaction, the residue was poured into ice-cold deionized water. The obtained white precipitate was filtered and washed with chloroform and 2 M NaOH solution. The aqueous layer was acidified with 5 M

HCl solution and then was extracted with ethyl acetate. The organic layers were combined and washed with brine solution, dried over anhydrous sodium sulfate, then filtered and concentrated. The crude product was purified by silica gel column chromatography using ethyl acetate as an eluent to afford compound **5.10** as a white solid (yield 77%). The compound was characterized by  $^1\text{H}$  and  $^{13}\text{C}$  NMR, which is in accordance with the literature report.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  7.90 (s, 2H), 2.35 (s, 6H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{ppm}}$  171.2, 158.8, 137.5, 135.2, 134.6, 21.7; ESI-MS calcd. For  $\text{C}_9\text{H}_9\text{NO}_4$   $[\text{M} + \text{H}]^+$ : 195.0532, found: 195.0944.<sup>35</sup>

**5.4.1.2.8. Synthesis of 3,5-bis(bromomethyl)-4-nitrobenzoic acid 5.11** – To the stirring solution of **5.10** (2 mmol), *N*-bromosuccinimide (NBS, 1.42 g, 8 mmol) in dry tetrachloromethane: *N,N*-dimethylformamide (DMF) (10: 1 v/v) was added azobisisobutyronitrile (AIBN, 30 mg), and the reaction mixture was refluxed for overnight. The reaction was monitored by TLC. After the completion of the reaction, the residue was poured into deionized water and then was extracted with ethyl acetate. The organic layers were combined and washed with saturated saltwater, dried over anhydrous sodium sulfate, then filtered and concentrated. The crude product was purified by silica gel column chromatography using DCM and methanol (10:0.8, v/v) as an eluent to afford **5.11** as white solid (yield 72%). **Characterization of compound 5.11:**  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta_{\text{ppm}}$  6.42 (s, 2H), 3.89 (s, 4H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{DMSO}-d_6$ )  $\delta_{\text{ppm}}$  167.8, 158.8, 132.9, 107.7, 107.2, 45.5, 39.8; HRMS (ESI) calcd. For  $\text{C}_9\text{H}_7\text{NO}_4\text{Br}_2\text{K}$   $[\text{M} + \text{H}]^+$ : 352.8815, found: 352.8830.

**5.4.1.2.9. Synthesis of (5<sup>2</sup>Z,7E)-3<sup>5</sup>-carboxy-1<sup>3</sup>,5<sup>3</sup>-didodecyl-1<sup>3</sup>,5<sup>3</sup>-dimethyl-32-nitro-1<sup>3</sup>H-1,5(1,2)-diindol-1-iuma-3(1,3)-benzenacyclooctaphan-7-en-1<sup>1</sup>-ium (Cy3L) - 1-iuma-3(1,3)-benzenacyclooctaphan-7-en-1<sup>1</sup>-ium (5.12)** – To a stirring solution of compound **5.7**, 0.784 mmol) in acetonitrile (100 mL) was added  $\text{K}_2\text{CO}_3$  (0.784 mmol), and the reaction mixture was continued to stir for 30 min at room temperature under  $\text{N}_2$  atmosphere. After that, 3,5-bis(bromomethyl)-4-nitrobenzoic acid (**5.11**, 0.784 mmol) was added and the reaction mixture was heated under reflux condition for 12 h. the reaction progress was monitored by TLC. After completion of reaction the unused solvent was removed reduced pressure yielded a viscous residue. The crude reaction mixture was purified by column

chromatography on silica gel using a gradient solvent system of DCM and methanol (10:0.5) to afford compound **5.Cy3L** as a brown gummy solid (yield 32 %).

**Characterization of compound 5.Cy3L:**  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta_{\text{ppm}}$  8.51 – 8.46 (m, 1H), 8.20 – 8.19 (m, 2H), 7.82 – 7.73 (m, 2H), 7.33 – 7.29 (m, 2H), 7.22 – 7.15 (m, 2H), 7.12 – 7.08 (m, 2H), 6.96 – 6.93 (m, 2H), 6.90 – 6.85 (m, 1H), 5.76 – 5.74 (m, 2H), 4.90 (s, 1H), 4.83 (s, 4H), 4.32 – 4.27 (m, 1H), 1.89 (m, 3H), 1.79 – 1.72 (m, 4H), 1.44 – 1.34 (m, 6H), 1.26 – 1.15 (m, 34H), 0.84 (t, 6H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3 + \text{DMSO}-d_6$ )  $\delta_{\text{ppm}}$  181.6, 168.3, 153.3, 152.1, 148.3, 146.4, 139.7, 138.3, 134.0, 133.1, 129.5, 127.6, 127.5, 125.1, 121.5, 67.4, 60.5, 53.3, 52.8, 44.3, 34.3, 32.5, 31.5, 29.2, 29.2, 29.1, 29.0, 28.4, 22.3, 14.0; HRMS (ESI) calcd. For  $\text{C}_{54}\text{H}_{74}\text{N}_3\text{O}_4$   $[\text{M} + \text{H}]^+$ : 829.5752, found: 829.5762.

**5.4.1.2.10. Synthesis of 5-amino-1,3,4-thiadiazole-2-sulfonamide (5.12)** - To a suspension of acetazolamide (2.77 mmol), in EtOH was added Conc. HCl 0.8 mL and reaction mixture was refluxed for 4 hr. The solvent was evaporated, saturated  $\text{NaHCO}_3$  was poured slowly into the reaction mixture, and the compound was extracted by ethyl acetate. Ethyl acetate layer was washed with brine and was dried over  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated to obtain compound **5.12** almost pure as a white solid (yield 94%). **Characterization of compound 5.12:**  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta_{\text{ppm}}$  8.08 (brs, 2H), 7.83 (brs, 2H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{DMSO}-d_6$ )  $\delta_{\text{ppm}}$  172.13, 158.34; HRMS (ESI) calcd. For  $\text{C}_2\text{H}_4\text{N}_4\text{O}_2\text{S}_2$   $[\text{M} + \text{H}]^+$ : 180.9848, found: 180.9872.

**5.4.1.2.11. Synthesis of 5-((tert-butoxycarbonyl)amino)pentanoic acid (5.13)** - To the stirring solution of 5-Aminovaleric acid (4.26 mmol) in dioxane:  $\text{H}_2\text{O}$  (2: 1, 25 mL), NaOH (4.26 mmol) was added under  $\text{N}_2$ -atmosphere and stirred the mixture at  $0^\circ\text{C}$  for 5 minutes. Then  $\text{Boc}_2\text{O}$  (6.68 mmol) was dissolved in dioxane:  $\text{H}_2\text{O}$  (2:1, 5 mL) and added dropwise. The resulting solution was stirred for 12 h at room temperature. The reaction progress was monitored by TLC. After maximum consumption of starting materials, the alkaline residue was dissolved in  $\text{H}_2\text{O}$  (20 mL) and washed thrice with EtOAc (3 x 30 mL). The aqueous phase was pH adjusted to pH 1-2 with 1M HCl and extracted with EtOAc (3 x 30mL). The organic phase was dried with  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated under reduced pressure. The obtained



white solid was almost pure and used without further purification. **Characterization of compound 5.13:**  $^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )  $\delta_{\text{ppm}}$  6.80 – 6.78 (m, 1H), 2.91 – 2.88 (m, 2H), 2.19 (t,  $J$  = 6 Hz, 2H), 1.46 (p,  $J$  = 6 Hz, 2H), 1.37 (s, 9H);  $^{13}\text{C}$  NMR (150 MHz, DMSO- $d_6$ )  $\delta_{\text{ppm}}$  174.8, 156.0, 77.8, 33.7, 29.4, 28.7, 22.2; HRMS (ESI) calcd. For  $\text{C}_{10}\text{H}_{19}\text{NNaO}_2$   $[\text{M} + \text{Na}]^+$ : 240.1206, found: 240.1243.

**5.4.1.2.12. Synthesis of tert-butyl (5-oxo-5-((5-sulfamoyl-1,3,4-thiadiazol-2-yl)amino)pentyl)carbamate (5.14)** - To the solution of compound **5.12** (2.22 mmol) in DMF was added triethylamine ( $\text{Et}_3\text{N}$ ) (2.22 mmol) and stir for 10 minutes. The reaction mixture cooled to 0 °C and sequentially added HBTU (4.4 mmol) and compound **5.13** (2.22 mmol), and the resulting reaction mixture was stirred for 12 h at room temperature. The reaction progress was monitored by TLC. After reaction completion, the reaction mixture was dissolved in ice-cooled water and washed with ethyl acetate thrice (3 x 30mL). The combined organic layers were dried over  $\text{Na}_2\text{SO}_4$  and evaporated under reduced pressure to the obtained crude mixture. The crude reaction mixture was purified by column chromatography on silica gel using a gradient solvent system of DCM and methanol (10:2) to afford compound **5.14** as a white solid (yield 82 %). **Characterization of compound 5.14:**  $^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )  $\delta_{\text{ppm}}$  7.95 (s, 1H), 7.26 (brs, 2H), 6.74 – 6.72(m, 1H), 2.90 (s, 2H), 2.87 – 2.85 (m, 2H), 2.74 (s, 2H), 1.95 (t,  $J$  = 6 Hz, 2H), 1.37 (s, 9H);  $^{13}\text{C}$  NMR (150 MHz, DMSO- $d_6$ )  $\delta_{\text{ppm}}$  178.5, 170.5, 162.3, 161.5, 155.5, 77.2, 45.6, 35.7, 30.7, 29.2, 28.2, 22.9; HRMS (ESI) calcd. For  $\text{C}_{12}\text{H}_{21}\text{N}_5\text{O}_5\text{S}_2$   $[\text{M} + \text{Na}]^+$ : 402.0882, found: 402.0876.

**5.4.1.2.13. Synthesis of 5-amino-N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)pentanamide (5.15)** - Compound **5.14** was dissolved in DCM and trifluoroacetic acid (TFA) (9: 1) mixture and stirred for 3 h at room temperature. The reaction progress was monitored by TLC. After maximum consumption of compound **5.14** the unused solvent was removed under reduced pressure and yielded a gummy mass which was washed with diethyl ether for several times. The solvent (diethyl ether) wash yielded the target product, which was used without further purification. **Characterization of compound 5.15:**  $^1\text{H}$  NMR (600 MHz,  $\text{D}_2\text{O}$  + DMSO- $d_6$ )  $\delta_{\text{ppm}}$  2.82 – 2.79 (m, 2H), 2.10 – 2.07 (m, 4H), 1.72 – 1.68 (m, 2H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{D}_2\text{O}$  +

DMSO- $d_6$ )  $\delta_{\text{ppm}}$  179.6, 172.8, 158.3, 39.3, 34.9, 23.6; HRMS (ESI) calcd. For  $\text{C}_7\text{H}_{13}\text{N}_5\text{O}_3\text{S}_2$   $[\text{M} + \text{H}]^+$ : 280.0538, found: 280.0574.

**5.4.1.2.14. Synthesis of 5.Cy3CAL** – To the solution of compound **5.15** (0.60 mmol) in DMF was added triethyl amine ( $\text{Et}_3\text{N}$ ) (0.60 mmol) and stir for 10 minutes. The reaction mixture cooled to 0 °C and sequentially added HBTU (1.2 mmol) and compound **5.Cy3L** (0.60 mmol) and the resulting reaction mixture was stirred for 12 h at room temperature. The reaction progress was monitored by TLC. After reaction completion, the reaction mixture was dissolved in ice cooled water and washed with ethyl acetate thrice (3 x 30 mL). The combined organic layers were dried over  $\text{Na}_2\text{SO}_4$  and evaporated under reduced pressure to obtained crude mixture. The crude reaction mixture was purified by column chromatography on neutral alumina using a gradient solvent system of DCM and methanol (10:1.5) to afford compound **5.Cy3CAL** as a red gummy solid (yield 77 %). **Characterization of compound 5.Cy3CAL:**  $^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )  $\delta_{\text{ppm}}$  8.48 – 8.45 (m, 1H), 8.20 (d,  $J = 6$  Hz, 1H), 7.91 – 7.77 (m, 1H), 7.41 (m, 1H), 7.30 – 7.29 (m, 2H), 7.18 – 7.15 (m, 2H), 7.09 – 7.08 (m, 2H), 6.96 – 6.93 (m, 2H), 5.76 (d,  $J = 12$  Hz, 2H), 4.90 (s, 1H), 4.83 (s, 4H), 4.32 – 4.30 (m, 1H), 3.11 (m, 2H), 2.20 – 2.11 (m, 2H), 1.89 (s, 3H), 1.78 – 1.75 (m, 2H), 1.67 – 1.61 (m, 4H), 1.43 – 1.34 (m, 8H), 1.27 – 1.17 (m, 39H), 0.84 (t, 6H); 189.9, 170.6, 164.5, 146.7, 136.2, 134.5, 134.1, 133.5, 129.8, 129.4, 129.3, 128.8, 127.9, 125.2, 122.1, 65.8, 64.2, 60.4, 41.7, 35.6, 32.7, 31.8, 31.7, 29.4, 29.4, 29.3, 29.1, 28.5, 27.9, 22.5, 22.4, 21.1, 14.4;  $^{13}\text{C}$  NMR (150 MHz, DMSO- $d_6$ )  $\delta_{\text{ppm}}$  189.9, 170.6, 164.5, 146.7, 136.2, 134.5, 134.1, 133.5, 129.8, 129.4, 129.3, 128.8, 127.9, 125.2, 122.1, 65.8, 64.2, 60.4, 41.7, 35.6, 32.7, 31.8, 31.7, 29.4, 29.4, 29.3, 29.1, 28.5, 27.9, 22.5, 22.4, 21.1, 14.4. ESI-MS calcd. For  $\text{C}_{61}\text{H}_{85}\text{N}_8\text{O}_6\text{S}_2\text{Na}$   $[(\text{M} + \text{Na})/2]^+$ : 556.2963, found: 556.4002.

**5.4.1.2.15. Characterization of compound 5.Cy3AAL:**  $^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )  $\delta_{\text{ppm}}$  9.82 (s, 1H), 8.45 (s, 1H), 8.22 – 8.21 (s, 1H), 7.84 – 7.81 (m, 2H), 7.41 (m, 1H), 7.30 – 7.29 (m, 2H), 7.18 – 7.16 (m, 2H), 7.09 – 7.06 (m, 2H), 6.96 – 6.94 (m, 2H), 6.47 – 6.45 (m, 2H), 5.77 – 5.75 (m, 2H), 4.85 (s, 2H), 3.17 – 3.12 (m, 2H), 2.12 – 2.10 (m, 2H), 1.90 (m, 3H), 1.79 – 1.77 (m, 4H), 1.68 – 1.61 (m, 4H), 1.40 – 1.34 (m, 8H), 1.23 – 1.13 (m, 36H), 0.84 (t, 6H);  $^{13}\text{C}$  NMR (150 MHz, DMSO- $d_6$ )  $\delta_{\text{ppm}}$  193.0, 183.0,



172.7, 170.9, 164.5, 154.5, 151.6, 147.1, 146.8, 144.0, 140.3, 136.2, 134.3, 134.1, 132.9, 129.8, 129.4, 128.3, 128.0, 125.2, 123.9, 122.2, 115.8, 109.0, 65.9, 60.4, 49.0, 45.8, 41.7, 35.6, 32.6, 31.7, 31.7, 29.4, 29.3, 29.3, 29.1, 28.5, 27.9, 26.5, 22.5, 22.4, 21.0, 14.4; MALDI-TOF-MS calcd. For  $C_{61}H_{85}N_8O_6S_2Na$   $[M + Na]^+$ : 1112.592, found: 1112.621.

#### 5.4.2. Spectroscopic studies of the amphiphile

**5.Cy3CAL** stock was prepared in chloroform and stored at  $-20^{\circ}C$ . The required amount of the compound from the stock solution was aliquoted in a glass vial and dried under reduced pressure to make the thin film. To this dried compound, phosphate buffer saline (PBS), pH 7.2, was added and vortexed to prepare the liposome. Liposome was further serially diluted in the buffer to get a concentration range from  $0.625\ \mu M$  to  $640\ \mu M$ . The UV-Vis spectra of the compound were measured both before and after NIR laser light treatment (15 min) using 1 mL quartz cuvette in evolution 201 UV-VIS spectrophotometer from 200 nm to 700 nm wavelength range. Similarly, the absorbance spectra of the compound were recorded in the presence of other natural lipid mixtures of DPPC/DPPS/Cholesterol (molar ratio of 60:20:20; 1 mM). The fluorescence properties of the amphiphile were also investigated both in buffer and in the presence of other natural lipids.

#### 5.4.3. Preparation of soluble aggregates

To prepare the thin film, the amphiphiles were dried under reduced pressure for 3 hours. After that, 500  $\mu L$  of PBS buffer, pH 7.2 was added into the thin film and heated to  $30-40^{\circ}C$  for 10 min. The solution was then vortexed well to mix the thin film. Finally, the solution was sonicated 3 times with 30 s of sonication followed by 30 s of cooling on ice cycles. A hand-held mini extruder with a polycarbonate membrane (diameter of 200 nm) was used to prepare small soluble aggregates at room temperature.<sup>4</sup>

#### 5.4.4. Morphological analyses of the nano-aggregates

The morphology of the aqueous soluble aggregates of the amphiphile **Cy3CAL** and (DPPC/DPPS/Cholesterol/**Cy3CAL**) was investigated both in the presence and absence of NIR (808 nm,  $1\ W\ cm^{-2}$  for 10 min.) light by the transmission electron

microscope (TEM) and FESEM. First, the aqueous solution of the compound was prepared by the above-mentioned method in PBS buffer, pH 7.2. For TEM analyses, a 10  $\mu$ L of 20  $\mu$ M solution was placed onto a carbon-coated copper grid and allowed to settle down for 1 minute. The grid was then carefully blotted with filter paper, and only a trace amount of the solution in the middle of the grid was kept. After that, the grid was allowed to dry for 10 minutes at 30 °C. Finally, 10  $\mu$ L of 2% uranyl acetate solution (in water) was added to the grid and allowed to dry for another 1 min at room temperature. The excess uranyl acetate solution was soaked off with tissue paper, and the grid was dried overnight at 30 °C. The images were collected using a JEOL JEM 2100 transmission electron microscope (operated at a maximum accelerating voltage of 200 kV). For FESEM analysis, the diluted solution was drop-casted on an aluminum foil-covered glass slide. The slide was allowed to dry overnight in a vacuum incubator. The dried sample on the glass slide was placed in the sample chamber of the FESEM instrument (Sigma 300, Zeiss), and images were collected using a voltage of 2-5 kV.<sup>4</sup>

#### 5.4.5. pH-dependent particle size and surface charge of the soluble aggregates

The soluble nano-aggregates were prepared according to the method as described in the earlier section in PBS buffer, pH 7.2. Both the zeta potential and hydrodynamic diameter of the soluble aggregates at different pH were measured using a Zetasizer Nano ZS90 (Malvern, Westborough, MA) instrument at 25 °C. Different pH buffer solutions were freshly prepared using isosmotic buffers consisting of 10 mM buffering agent and 10 mM salt to vary the pH range (pH 3 to pH 9.0) and used for the measurements. Citric acid and trisodium-citrate for pH 3.0–6.5; 3-(*N*-morpholino)propanesulfonic acid (MOPS) for pH 7.0 and tris(hydroxymethyl)aminomethane (Tris)–HCl were used for pH 7.5–8.5.

#### 5.4.6. Phase transition temperature measurements

Described at section 2.4.6.

#### 5.4.7. NIR light-induced cleavage of the amphiphile

Light cleavable property of the compound **Cy3CAL** was monitored by the <sup>1</sup>H NMR analyses. To a solution of compound **Cy3CAL** in DMSO-*d*<sub>6</sub>, a NIR laser diode

(808 nm) of power density ( $1 \text{ W cm}^{-2}$ ) was placed at 3 cm above the liquid surface and irradiated for 3, 5, 7, 10, 13, 15 minutes, and the NMR spectra were recorded of corresponding time.

#### 5.4.8. Doxorubicin encapsulation and release efficiency measurement

To measure the Dox release profile from vesicles, a dialysis-based method was used according to the reported procedure.<sup>4, 19</sup> To a glass vial, prepared the multilayers amphiphile and added 1 mL Dox solution in PBS (final concentration lipid and DOX are 40 mM and 1 mM, respectively). Then, kept the solution for a few minutes for hydration; after that, the solution was vortexed well for 10-15 minutes and then sonicated, as mentioned earlier. The unencapsulated free Dox was separated by centrifuge followed by three times washing with the same PBS buffer, pH 7.2. Vesicles containing Dox were added into a D-Tube™ Dialyzer (Millipore Sigma, molecular weight cut-off 10 kD). After that, two types of the experiment was set up; in the first experiment, a NIR laser diode was set at 3 cm above the liquid surface and irradiated for 15 minutes, and another solution was irradiated batch-wise manner (five cycles of 3 minutes light irradiation and 20 minutes kept in the dark). Then, the tubes were sealed and kept in a reservoir containing 10 mL of the same buffer. At scheduled time points, 20  $\mu\text{L}$  of the reservoir solution was injected through analytical HPLC (C18, 15 cm  $\times$  4.6 mm, 2.7  $\mu\text{m}$ ) to monitor the release of free Dox. Concentrations of released Dox in the supernatant were determined by measuring the area of the chromatogram at 254 nm. Water and acetonitrile solvent was used as mobile phase with a flow rate of 0.5 mL/minute. To measure the loaded drug, the Dox@Cy3CAL and Dox@(PC/PS/Chol/Cy3CAL) was dissolved in acetonitrile and recorded through HPLC.

#### 5.4.9. Cell culture

The MCF-7 and MDA-MB-468 (breast cancer) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and penicillin-streptomycin solution (GIBCO; 100 U/ml penicillin and 100  $\mu\text{g/ml}$  streptomycin). HEK-293 (Human embryonic kidney cells) cultured in DMEM supplemented with the above-mentioned media. Cells were maintained in a

humidified atmosphere of 5% CO<sub>2</sub> at 37 °C in a CO<sub>2</sub> incubator. In all experiments, the medium was changed daily with complete media.

#### 5.4.11. Determination of cell viability using MTT assay

The MCF-7 and HEK-293 cells were cultured, as mentioned earlier. For the cell viability study, both the cell lines were seeded in 96-well flat-bottomed plates at a density of  $2 \times 10^4$  cells per well and incubated for 24 hours in complete media in a CO<sub>2</sub> incubator. Thereafter, cells were treated with different concentrations of **Cy3CAL**, free Dox, and Dox@ **Cy3CAL** (in the presence and absence of 808 nm light according to the experiment) for additional 48 hours. The cell viability was then assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma-Aldrich) assays. Briefly, media was discarded, and cells were treated with MTT (final concentration: 0.5 mg/ml) for 2 hours at 37 °C in a CO<sub>2</sub> incubator, and the resulting purple-colored formazan crystals were dissolved in 200 µL of DMSO. Cell viability was then measured using Multiscan GO (Thermo Scientific) at 570 nm. Cell viability (%) = (absorbance intensity (sample)) / (absorbance intensity (control)) × 100%.

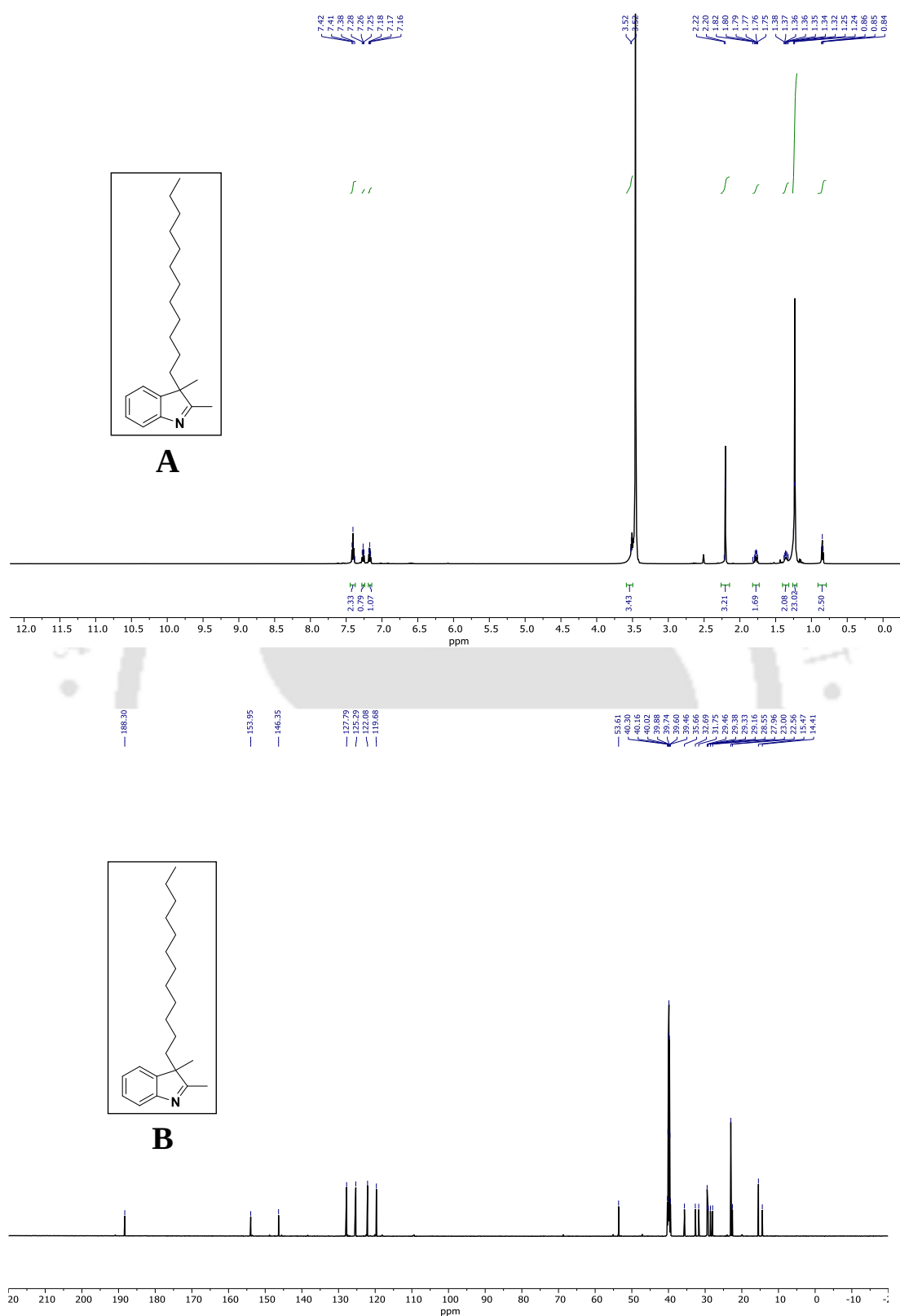
#### 5.4.12. Determination of cellular uptake efficiency using flow cytometry

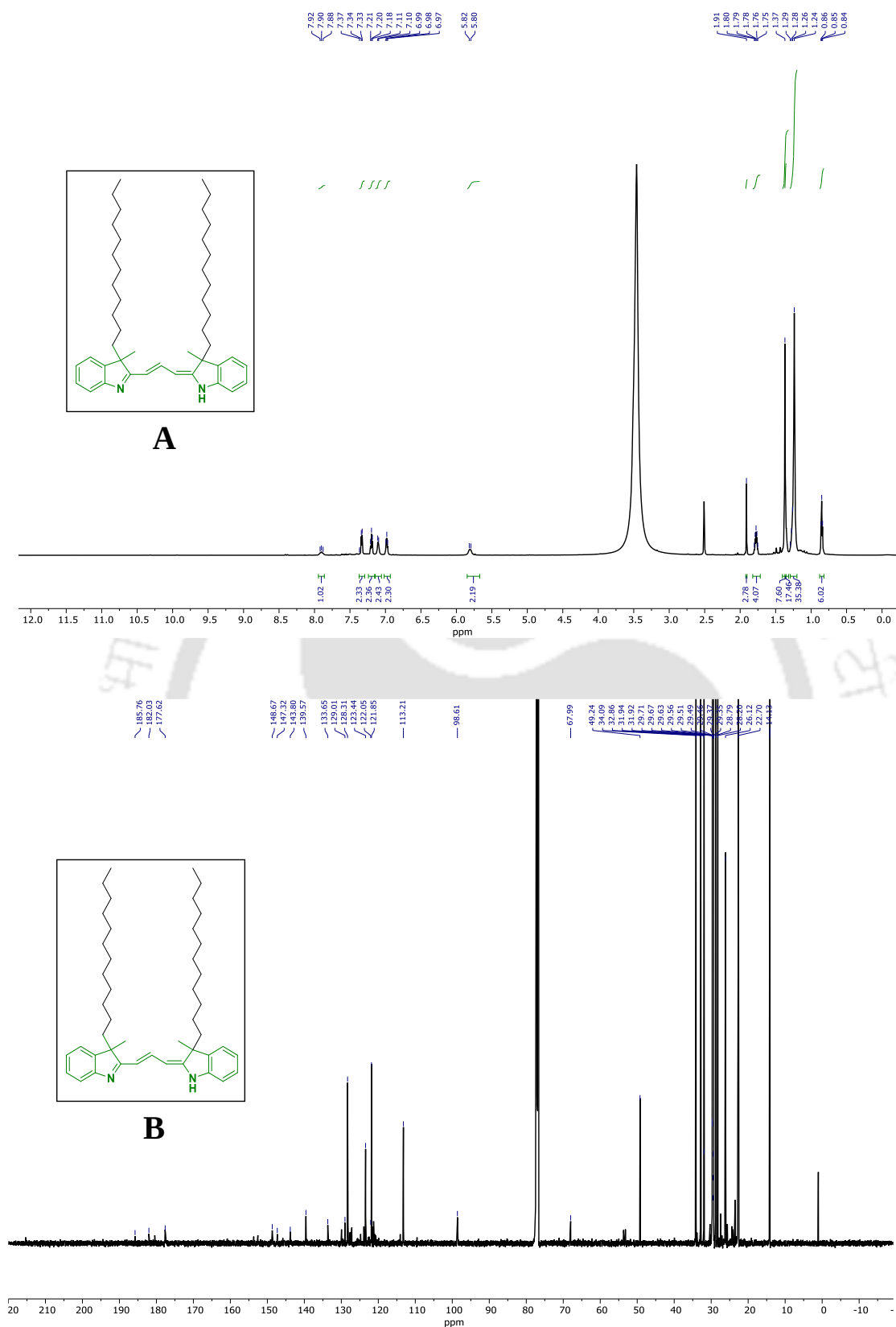
The MCF-7 cells were cultured, as mentioned earlier. Cells were seeded in 6-well plates at a density of  $2 \times 10^5$  cells per well and incubated for 24 hours in complete media. The cells were then treated with different entities (Dox, Dox@ **Cy3CAL**) in the absence and presence of NIR light (808 nm, 1 W cm<sup>-2</sup>, 10 min.) in 1% FBS containing media for 8 hours at 37 °C. Next, the cells were washed twice with PBS (GIBCO), trypsinized (0.05%; GIBCO) for 2 minutes at 37 °C followed by 5 min centrifugation at 800 rpm at 4°C. The supernatant was discarded, and cell pellets were re-suspended in 300 µL of PBS (supplemented with 2% FBS). Finally, the solutions were kept on ice, and the drug uptake by the MCF-7 cells was analyzed by flow cytometry analysis using BD FACS Calibur Flow Cytometer (BD Biosciences). Data analysis was performed by the FlowJo X Flow Cytometry software.

#### 5.4.13. Determination of cellular uptake using microscopy analysis

The MCF-7 cells were cultured, as mentioned earlier. For microscopy analysis, cells were seeded in 6-well plates at a density of  $1.5 \times 10^5$  cells per well, each well

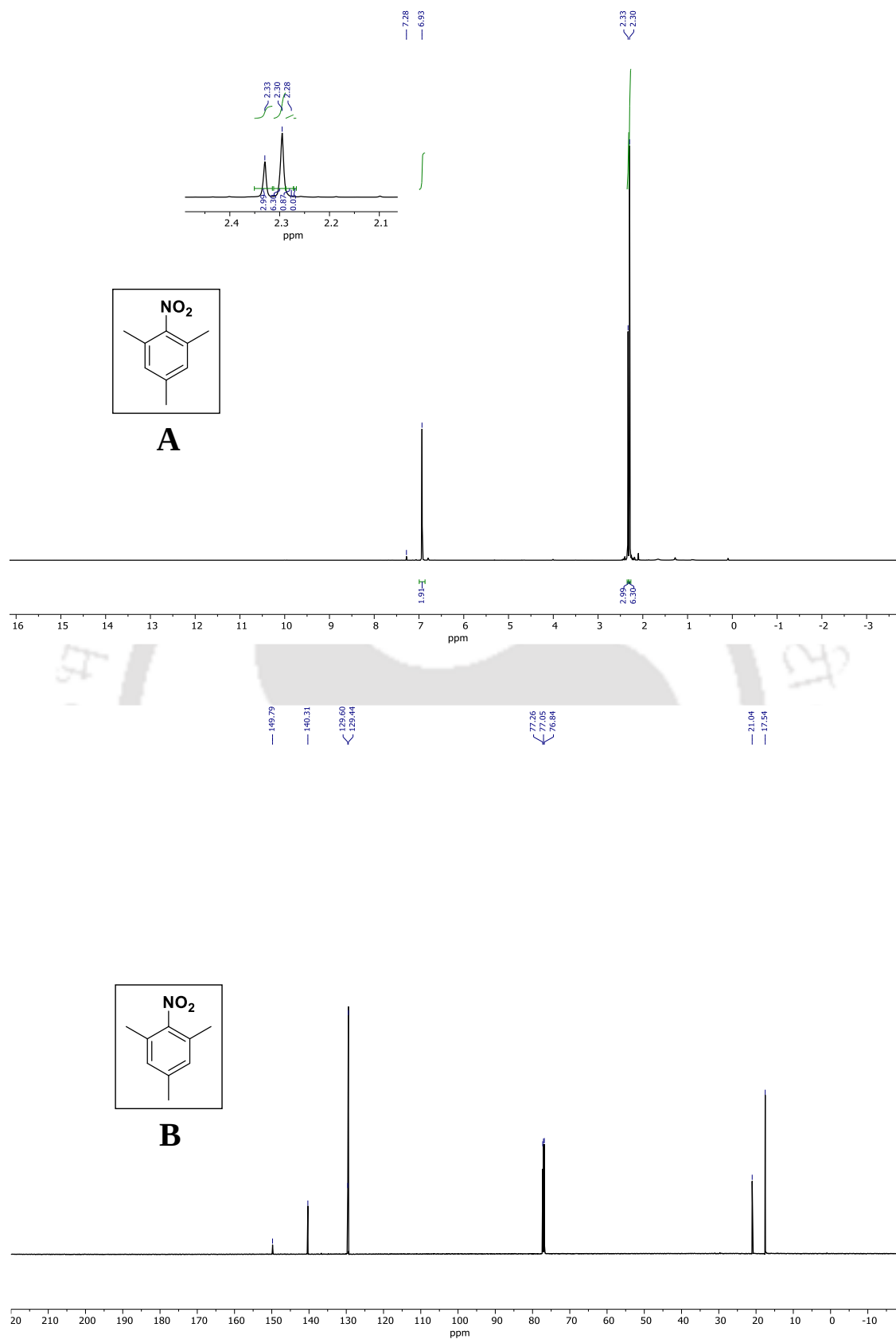
containing a sterile 22 mm square coverslip (pre-coated with 0.1% gelatin) in the bottom and incubated overnight in complete media in a CO<sub>2</sub> incubator. Cells were then treated with Dox@ **Cy3CAL** in the presence and absence of light for 10 minutes and further incubated for 8 hours in 1% FBS-containing media. Post-treatment, cells were washed with PBS buffer and fixed with 4% formaldehyde at 37 °C for 10 minutes. Coverslips were then washed thrice with PBS and treated with 0.1% Triton X-100 for 5 minutes at 37 °C. Cells were again washed thrice with PBS buffer. To counterstain the nuclei, DAPI (5 µg/mL) was added and incubated for 5 minutes. Cells were then washed thrice with PBS. Subsequently, coverslips were taken out from the wells and mounted over a glass slide. The slides were then allowed to dry for 15 mins. Finally, the microscopic analysis was performed using a confocal laser scanning microscope (CLSM; Leica TCS SP8;  $\lambda_{\text{ex}} = 488 \text{ nm}$ ,  $\lambda_{\text{em}} = 560\text{--}630 \text{ nm}$  for Dox and  $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}} = 440\text{--}480 \text{ nm}$  for DAPI;  $\lambda_{\text{ex}} = 535 \text{ nm}$ ,  $\lambda_{\text{em}} = 570 \text{ nm}$  of **Cy3CAL**).

5.4.13.  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of synthesized compoundsFigure 5.12.  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 5.6.

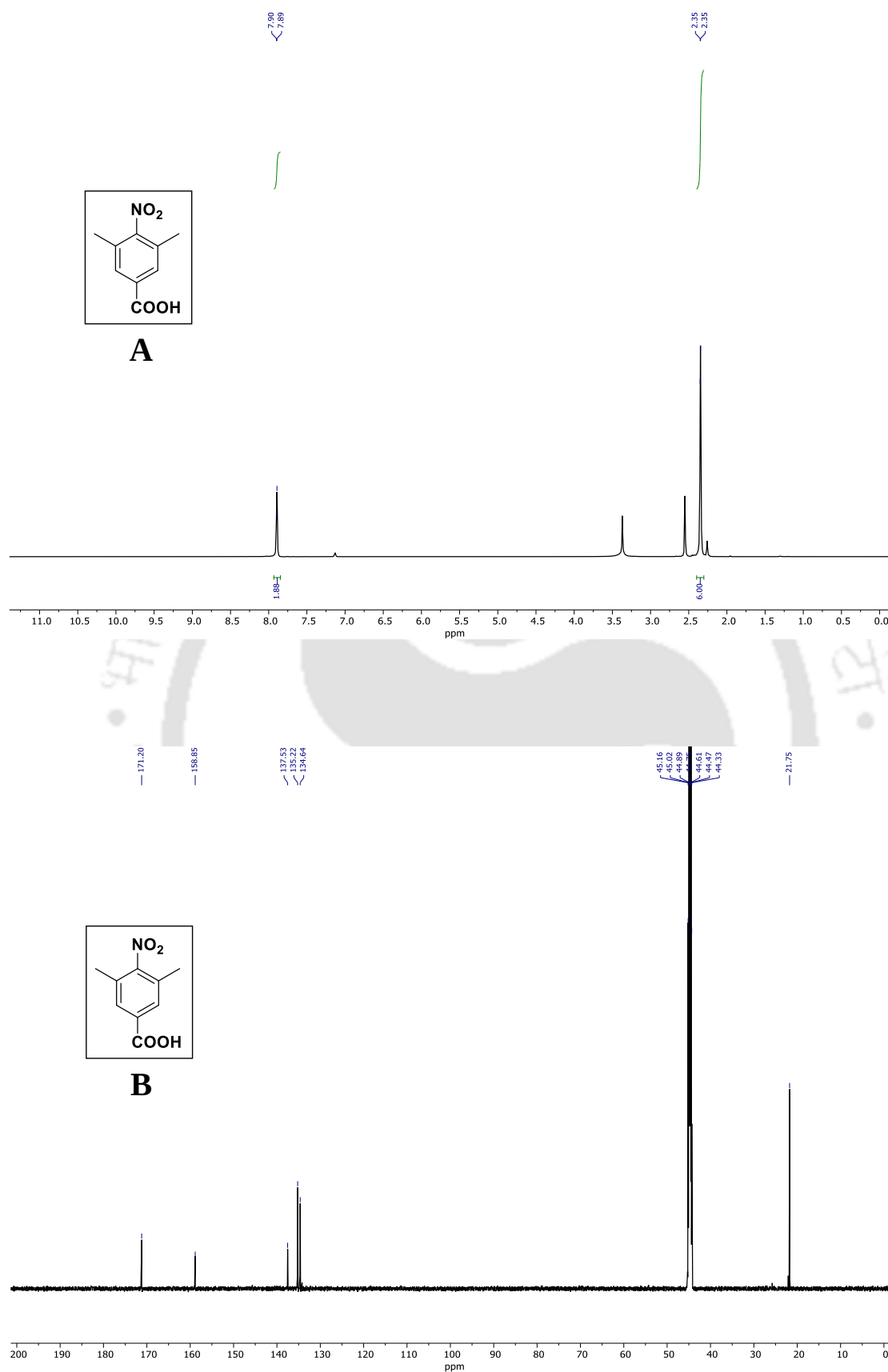


**Figure 5.13.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 5.7.

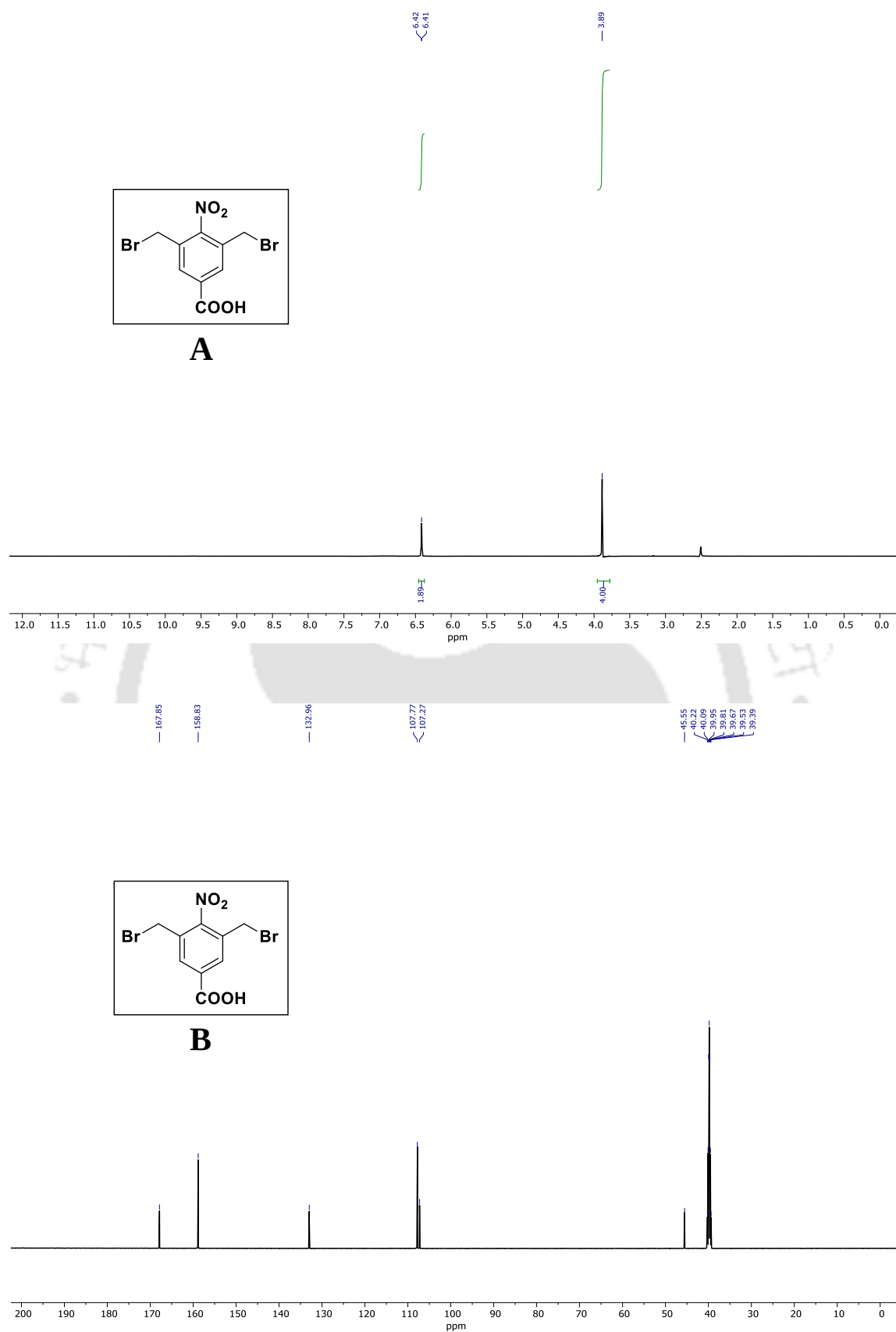




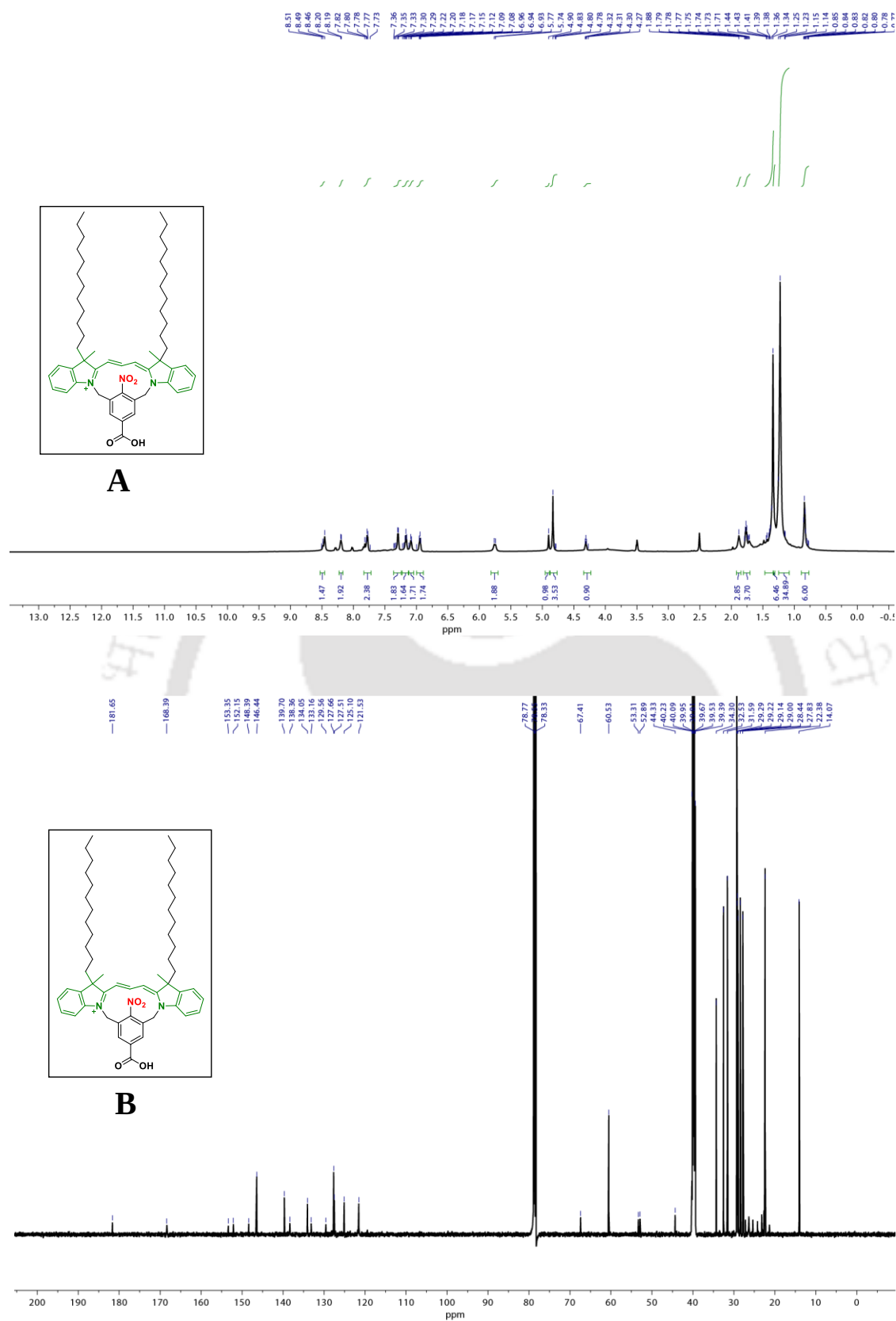
**Figure 5.14.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 5.9.

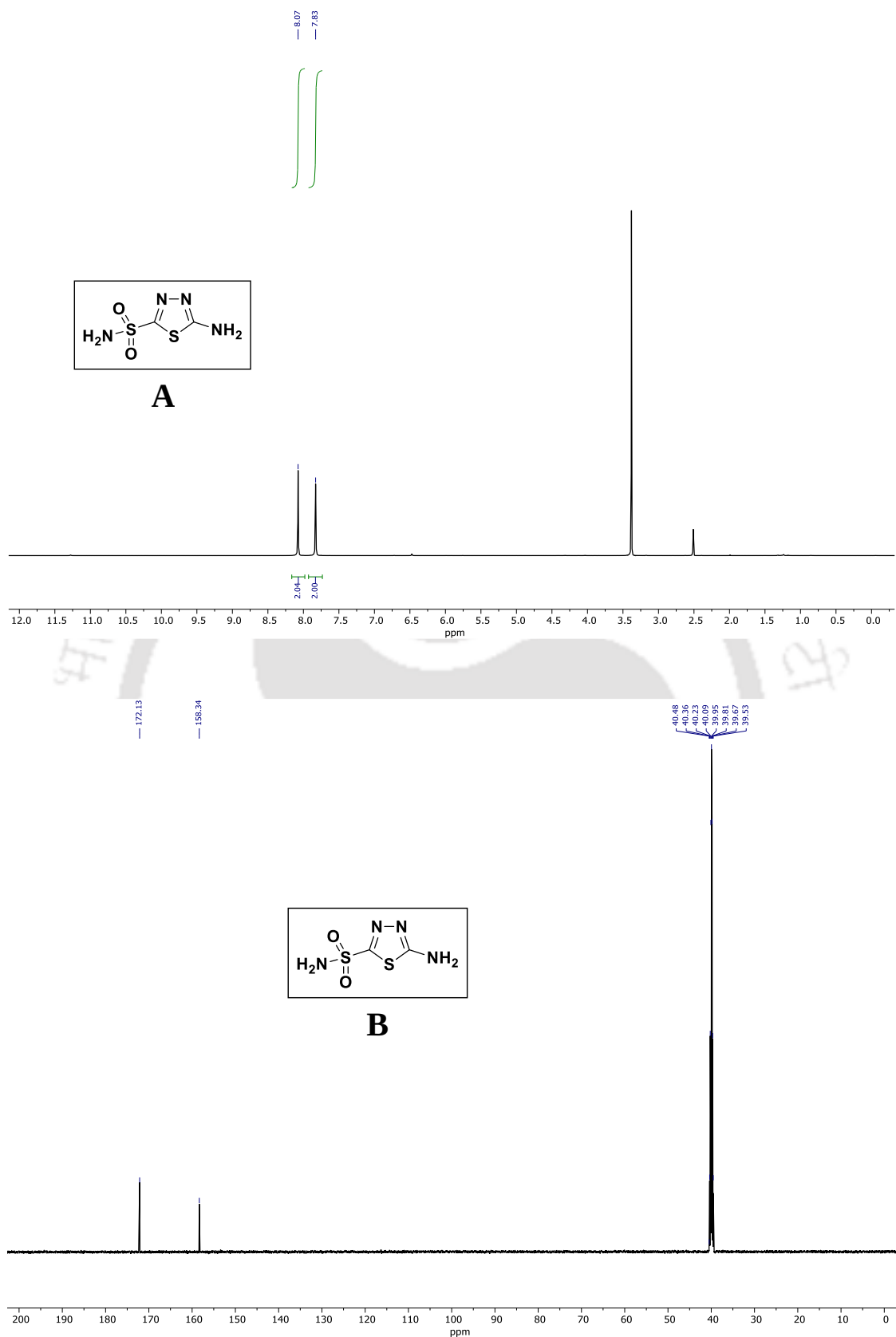


**Figure 5.15.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 5.10.

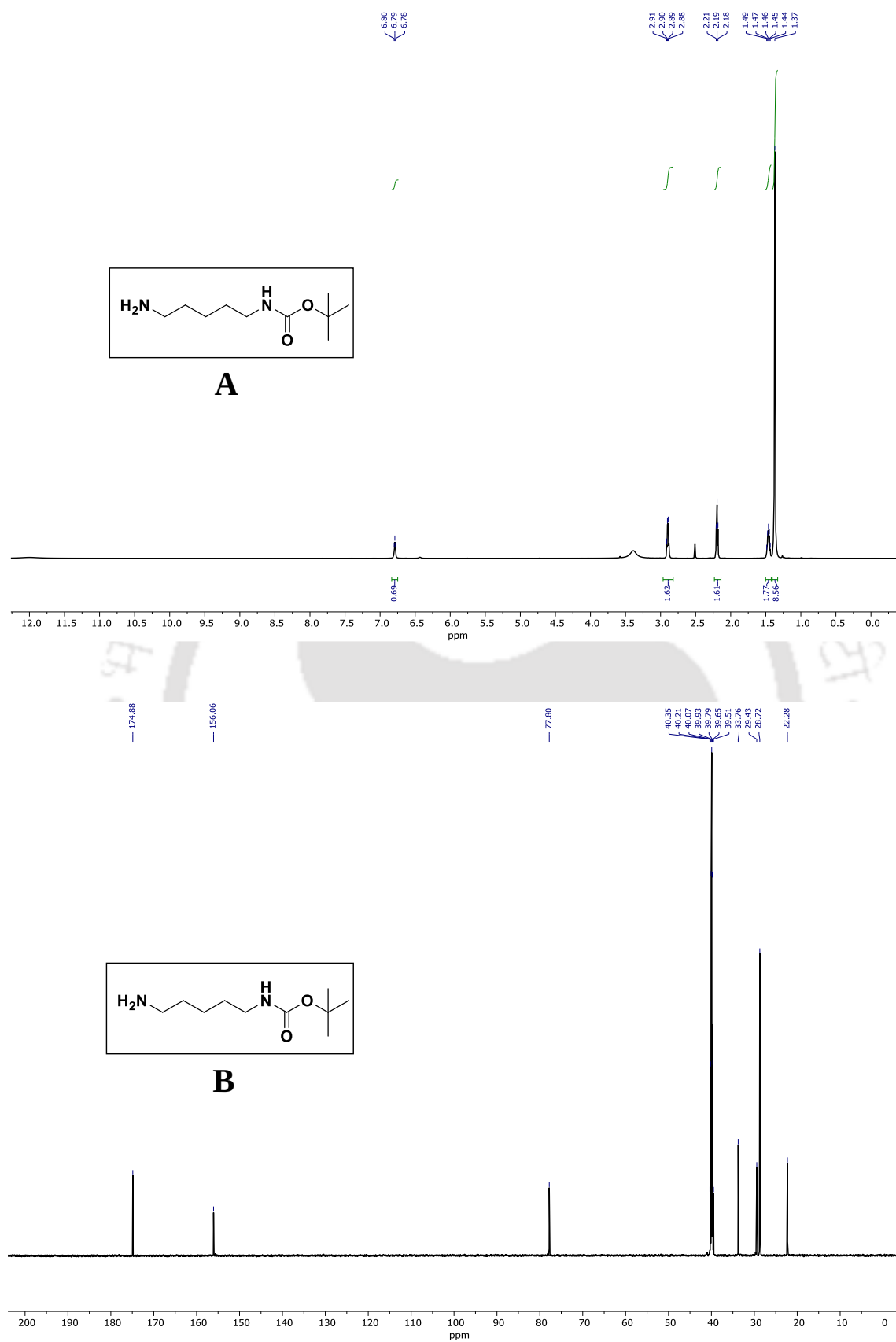


**Figure 5.16.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 5.11.

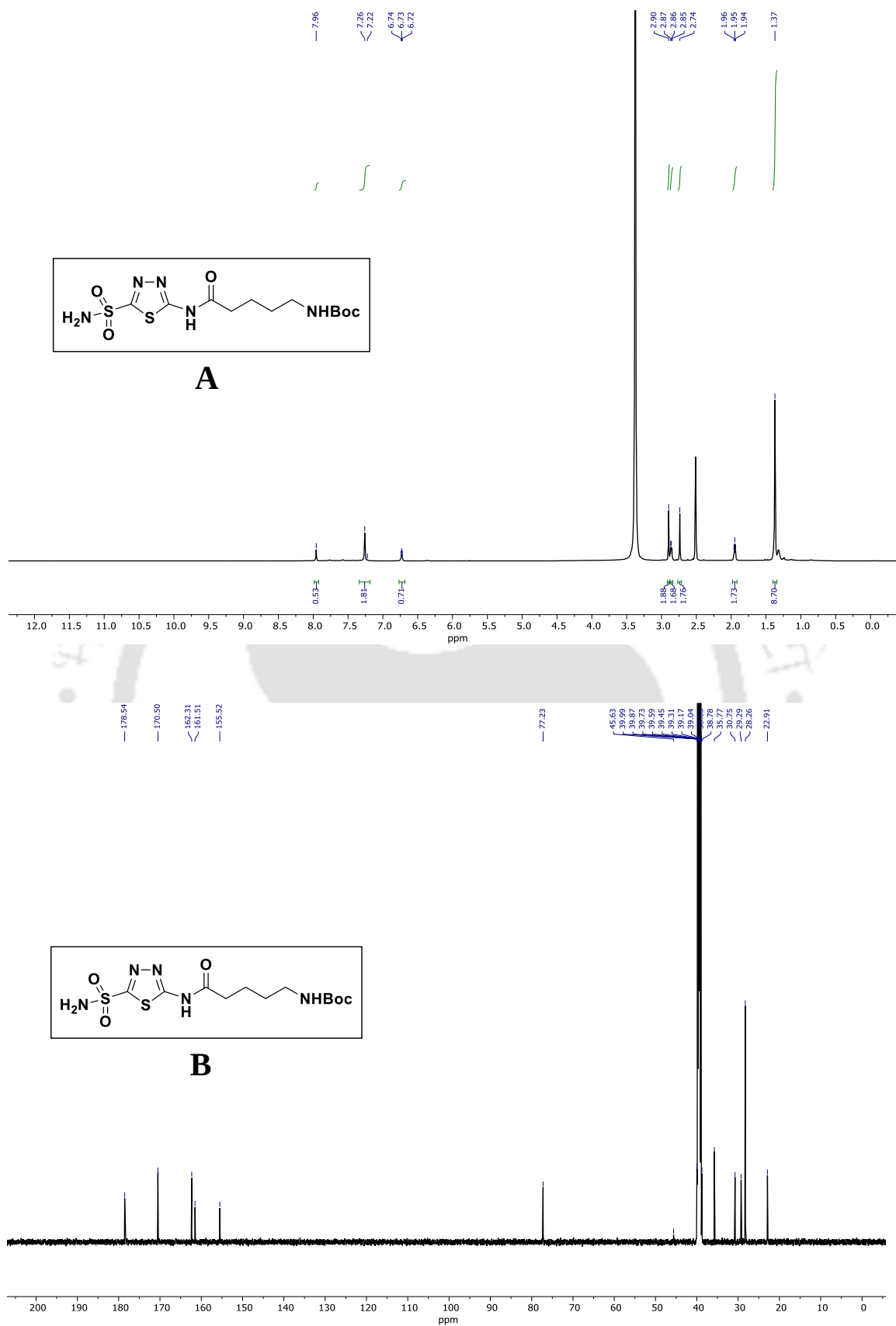




**Figure 5.18.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 5.12.

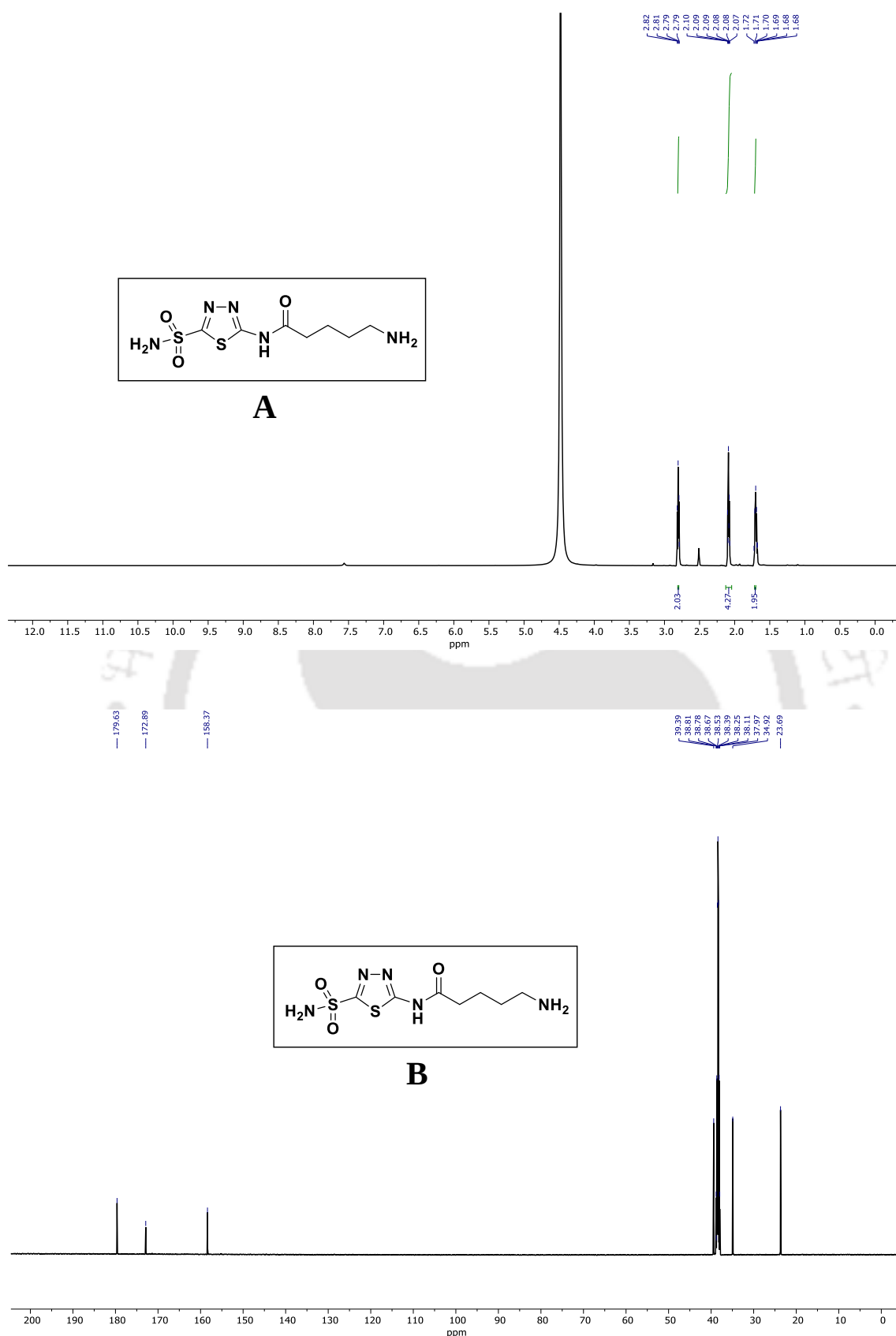


**Figure 5.19.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 5.13.

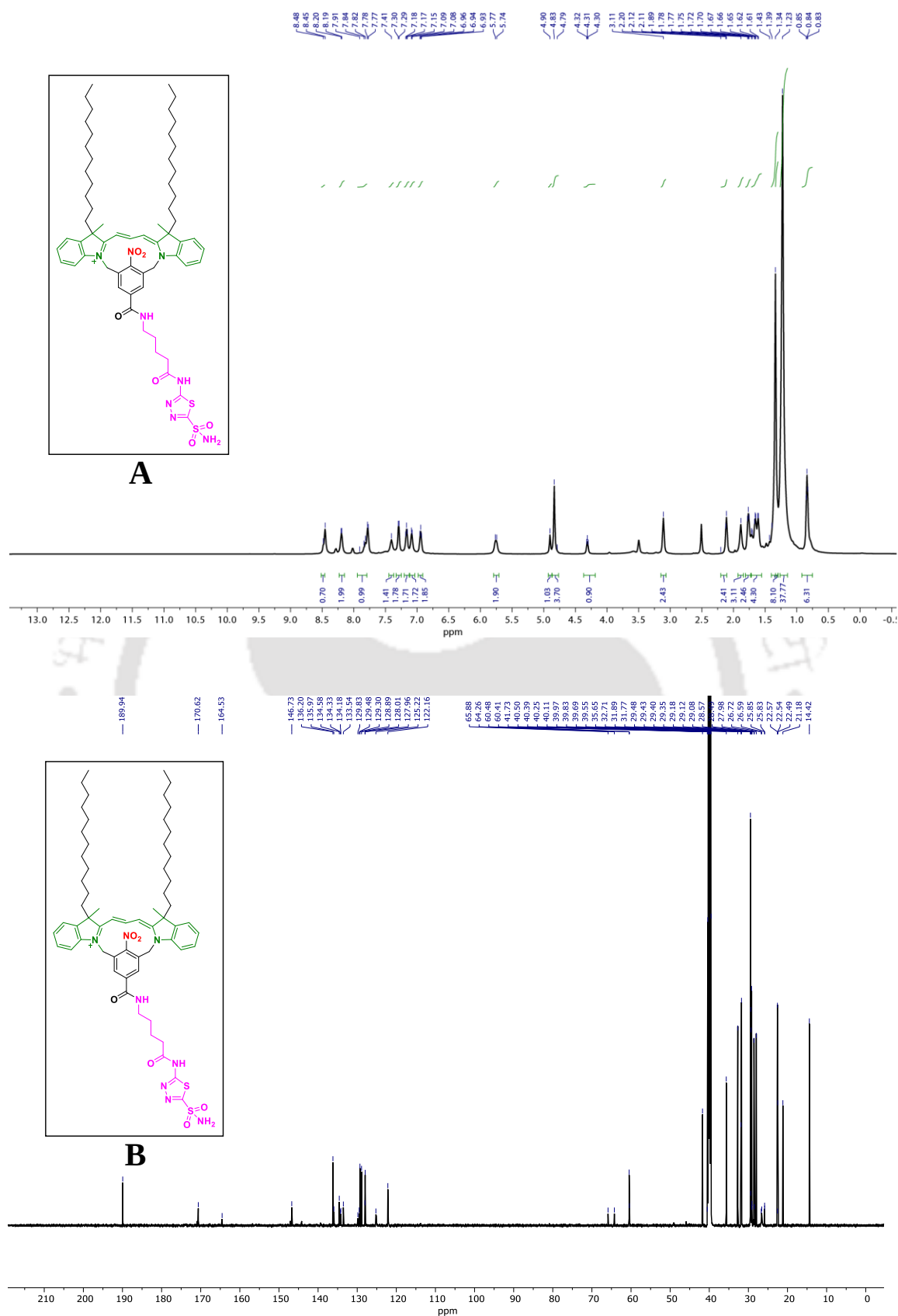


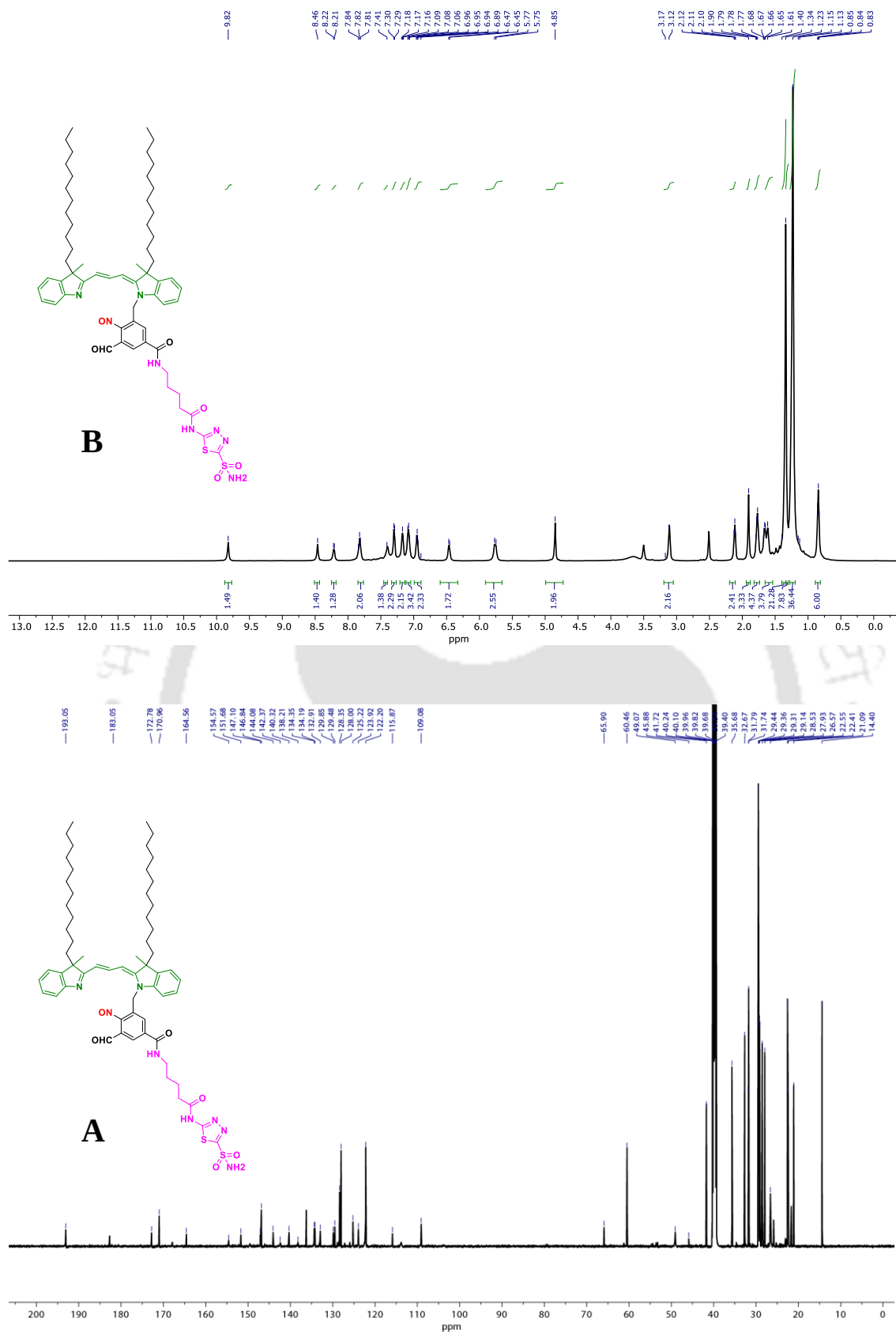
**Figure 5.20.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 5.14.





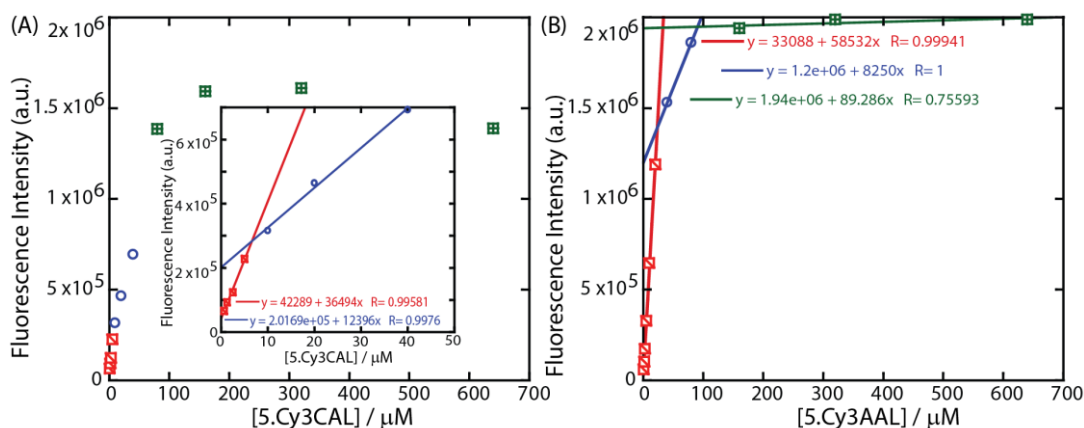
**Figure 5.21.**  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) spectra of compound 5.15.





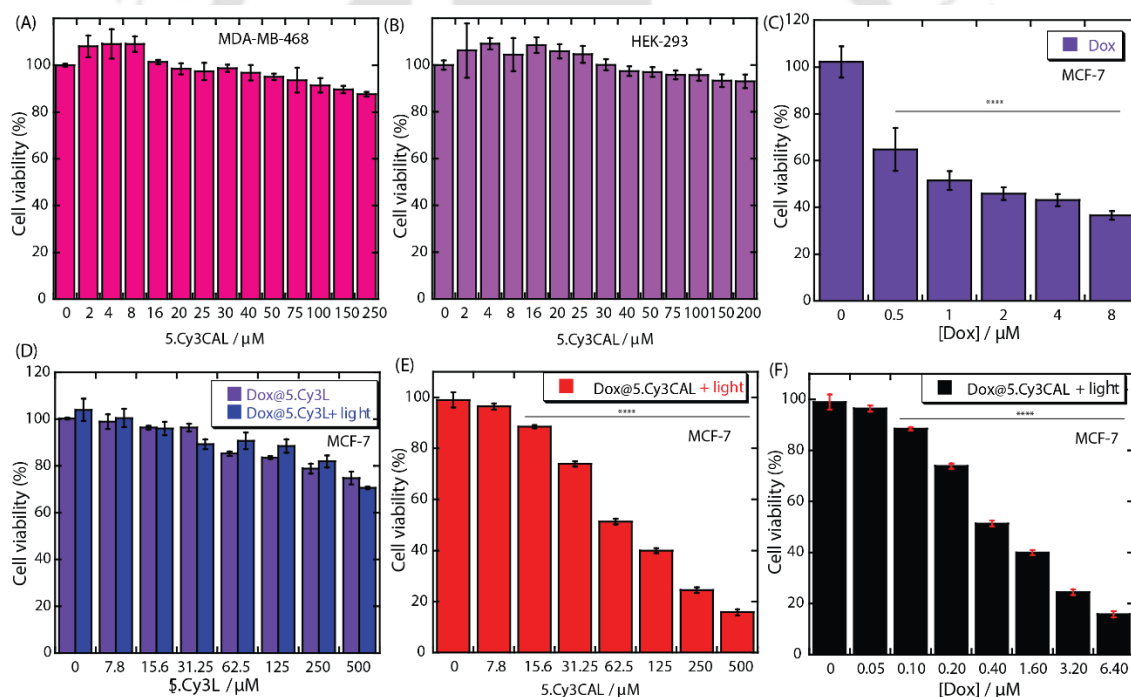
**Figure 5.23.** <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) spectra of compound 5.Cy3AAL.

## 5.4.14. Fluorescence based CAC measurement



**Figure 5.24.** Measurement of the critical aggregation constant of 5.Cy3CAL and 5.Cy3AAL.

## 5.4.15. Cell viability



**Figure 5.25.** Viabilities of MDA-MB-468 (A) and HEK-293 (B) cells in the presence of 5.Cy3CAL at different concentrations. Viabilities of MCF-7 cells in the presence of Dox at different concentrations (C). Viabilities of MCF-7 cells in the presence of 5.Cy3CAL (before and after light treatment for 15 min) at different concentrations (D). Viability of MCF-7 cells in the presence of Dox@5.Cy3CAL after light treatment with respect to the concentration of 5.Cy3CAL (E) and DOX (F). NIR laser light (808 nm, 1 W cm<sup>-2</sup>) source was used irradiation of the samples for 15 minutes

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## 6. Thesis Conclusion

In this thesis, I have successfully described the role of synthetic liposomes for the delivery of gene and drug molecules. Concise literature on various applications of lipid-based drug delivery systems has been discussed in **Chapter 1**. Lipid synthesis via sunlight-mediated thiol-yne click reaction in an aqueous environment has been explored (**in Chapter 2**), and few lipids showed promising DNA transfection efficiency with a very low cytotoxicity effect. After that, my work was based on developing stimuli-responsive lipid molecules as drug carriers for precise delivery. GSH-responsive sulfonium-based amphiphiles have been developed (**in Chapter 3**) for cancer cell-selective delivery of the anti-cancer drug Dox. We explored their physicochemical properties and drug loading efficacy, deliverability to the cancer cell, and the inherent antibacterial activity of the vehicle. Although the sulfonium-based lipids have been active carriers, the system failed when to the insignificant concentration differences of GSH between cells. A series of photoresponsive macrocyclic lipids have been developed to overcome the limitations of externally controllable drug delivery systems (**Chapter 4**). Detailed biophysical and *in vitro* studies were also carried out to prove the potential delivery system. To improve the delivery efficacy of photoresponsive lipids, the focus has been given to develop a novel photoresponsive fluorescent amphiphile with cancer cell targetable ligand (**in Chapter 5**) for early diagnostic image-guided delivery. Overall, this thesis dealt with the development of various novel lipid molecules and explored their biological applications.

## 7. Future Prospects

The science of lipid-based drug delivery systems is currently among the fascinating research areas. Over the last few decades, significant advancement has been made in the field of liposome-based drug delivery systems. As outlined in the various chapters above, cancer appears to be one of the examples where both its diagnosis and therapy have benefited from nonmedical strategies. The use of nano-drug drug delivery systems to deliver accurate doses to the affected cancerous cells without disturbing the physiology of the normal cells is certainly the trend that will remain in the future arena of research and development for decades to come.

In spite of the overwhelming understanding of the prospect of nanomedicine and nano-drug delivery systems, its actual impact on the healthcare system, even in cancer therapy/diagnosis, is still very limited because many key fundamental attributes remain unknown. The vital biological markers that allow absolute targeting of cancerous cells without altering the normal cellular process are one main future research area. The application of nanomedicine will advance with increasing knowledge of diseases at the molecular level, or the identification of suitable biomarkers will open up avenues for new diagnosis/therapy. Hence further research would be essential for the broader application of nanomedicine and nano-drug delivery systems. With the growing interest in precise medicines and diagnosis, the future for the smart and multi-centered approach to lipid-based nano-drug delivery technology looks bright. Motivated by all these therapeutic prospects, a diverse range of novel lipid molecules have been developed throughout this thesis. However, the selective delivery between a normal cell and a cancer cell is still a substantial hurdle. In addition, the heterogeneity and metamorphism behavior of cancer cells make an immense challenge to cancer cell-targeted drug delivery and demand better understanding to develop a smart carrier. The use of passive or active multi-targeted drug delivery systems could be a practical approach to anticancer therapeutics. The development of smart multi-functional lipids with more than one cancer-targeting ligand can be explored in the future.

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Cite This: ACS Omega 2020, 5, 735–750

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Article

## Sunlight-Mediated Thiol–Ene/Yne Click Reaction: Synthesis and DNA Transfection Efficiency of New Cationic Lipids

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### A stimuli-responsive anticancer drug delivery system with inherent antibacterial activities

S. Dey, A. Patel, K. Raina, N. Pradhan, O. Biswas, R. P. Thummer and D. Manna, *Chem. Commun.*, 2020, **56**, 1661  
DOI: 10.1039/C9CC08834B

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**Photoresponsive transformation from spherical to nanotubular assemblies: anticancer drug delivery using macrocyclic cationic gemini amphiphiles**

S. Dey, S. Chatterjee, A. Patel, N. Pradhan, D. Srivastava, N. Patra, A. Bhattacharyya and D. Manna, *Chem. Commun.*, 2021, **57**, 4646 DOI: 10.1039/D1CC01468D

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## Publications

1. **Dey, S.**; Chatterjee, S.; Patel, A.; Pradhan, N.; Srivastava, D.; Patra, N.; Bhattacharyya, A.; Manna, D.\* "Photoresponsive transformation from spherical to nanotubular assemblies: anticancer drug delivery using macrocyclic cationic gemini amphiphiles". *Chem. Commun.*, **2021**, 57(38), 4646-4649.
2. **Dey, S.**; Patel, A.; Raina, K.; Pradhan, N.; Biswas, O.; Thummer, R.P.; Manna, D.\* "A stimuli-responsive anticancer drug delivery system with inherent antibacterial activities". *Chem. Commun.*, **2020**, 56(11), 1661-1664.
3. **Dey, S.**; Gupta, A.; Saha, A.; Pal, S.; Kumar, S.; Manna, D.\* "Sunlight-Mediated Thiol–Ene/Yne Click Reaction: Synthesis and DNA Transfection Efficiency of New Cationic Lipids". *ACS omega.*, **2019**, 5(1), 735-50.
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