

Boronic Acid Materials for Carbohydrate Recognition and Enhanced Cellular Delivery

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

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August 2019

Acknowledgements

First and foremost, I would like to express my sincere thanks to my advisor Dr. Michael Best. I am deeply grateful for his encouragement and support throughout my PhD study. His passion towards fundamental science, warm and genuine personality are something I've always tried to learn from. I was very lost in my first two years in grad school, Dr. Best was patient and guided me through hard time. He is the first one I want to share good news with when I have some exciting results in research. I consider myself very lucky to have the opportunity working and studying in his lab.

Special thanks to Dr. David Baker, Dr. Bhavya Sharma and Dr. Todd Reynolds for serving as my dissertation committee. I greatly appreciate their insightful suggestions throughout the research and dissertation preparation. It would have been impossible to complete this work without their tremendous help. The impact of their academic excellence and strong working ethics is one of the greatest gifts I have received in my life.

Thanks to the faculty, staff and graduate students in the Chemistry Department at UTK for providing a positive and encouraging environment. Thanks to Dr. Steven Neal and Dr. Lauren Anderson for their help with my TA works. Thanks to Dr. Carlos Steren for his help in NMR analysis.

I would like to thank Drs. Shiqiang Zhang and Seung Baek from the College of Veterinary Medicine in UT, for their assistance in culturing cells for my first project. I would like to thank Drs. Daiane Alves and Francisco Barrera from the UTK BCMB Department, for their help in biological studies for my later projects. Special thanks to my

wonderful summer REU undergrad Shelby D. Hill. Thanks to Dr. John Dunlap from UTK Advanced Microscopy and Imaging Center in UT for his help with STEM studies.

With a special mention to all colleagues in the Best Lab past and present. Working with them has been the unforgettable experience in my life. I am especially thankful for the help from Dr. Shahrina Alam, Dr. Stuart Whitehead, Dr. Tanei Ricks, Dr. Sam Mattern-Schain, Dr. Adam Carr, Dr. Alex Fisch, Zhengsu Yu, Alexa Watson, Jinchao Lou, Sara Barker, Christelle Ancajas, Ruhani Sagar, Megan Qualls, Matt Johnson and many other colleagues.

I would like to thank my family, especially my dad Xiong Zhang, my mom Shen Zhou and my husband Dr. Shutang Steve You for their love and support. You guys mean the whole world to me.

This work is dedicated to my beloved grandpa Weijun Zhou.

Abstract

Cell surface glycans, typically in the form of glycoproteins or glycolipids, are commonly aberrant in diseases and can serve as biomarkers that facilitate diagnostics, selective cellular labeling and delivery. Boronic acids have been extensively utilized for the detection and separation of carbohydrates through reversible formation of boronate esters in the binding of diol moieties on glycans. However, the biological application of this sensing group is challenged by its relatively low binding affinity in aqueous media. Through the research described in this dissertation, we designed and synthesized boronic acid materials that can enhance its avidity towards glycan through multivalent binding interactions to achieve biological applications including glycoprotein detection and cellular delivery.

In the second chapter of this dissertation, a boronic acid microplate assay for sensitive and high-throughput detection of the cancer-associated glycoprotein mucin-1 was developed. For this purpose, a multivalent surface for glycoprotein recruitment and detection was achieved by immobilizing biotinylated boronic acid onto streptavidin-coated microplates. In the third chapter, novel boronic acid lipids were synthesized and incorporated into liposomes as a drug delivery platform. We validated carbohydrate-responsive liposome efficacy by demonstrating dose-dependent release of encapsulated hydrophobic or hydrophilic fluorophores upon titration with the polysaccharide heparin. Cellular delivery and labeling were visualized by confocal microscopy indicating a

dramatic enhancement in fluorescence signal, showcasing the prospects of boronic acid lipids for drug delivery. In the last chapter, bisboronic acid lipids containing a small library of spacers of varying length between two boronic acid units have been synthesized to study selective binding interactions towards sialic acids. Enhanced binding affinity towards *N*-acetylneuraminic acid and the sialic acid-rich glycoprotein porcine mucin were observed using bisboronic acid lipids, particularly when using a *meta*-xylene spacer. This was detected via dye displacement competitive fluorescence assays as well as through liposomal triggered release studies. Finally, dose-dependent fluorescence cellular labelling was visualized using bisboronic acid liposomes. Collectively, these studies show that the incorporation of boronic acids into complex architectures including microplate surfaces and liposomes is effective for advancing applications such as the detection of disease-associated carbohydrates in complex biological samples and the delivery of therapeutic agents to cells.

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List of abbreviations

BA	Boronic acid
TF	Thomsen–friedenreich
SLe ^x	Sialyl lewis x
API	Active pharmaceutical ingredient
HPMA	<i>N</i> -(2-hydroxypropyl)-methacrylamide
PEG	Poly(ethylene glycol)
MLV	Multilamellar vesicles
LUV	Large unilamellar vesicles
SUV	Small unilamellar vesicles
LPC	Lyso-phosphatidylcholine
PE	Phosphatidylethanolamine
EWG	Electron- withdrawing group
PA	Phosphatidic acid
POPA	1-Oleoyl-2-palmitoyl-phosphatidic acid
POPC	1-Oleoyl-2-palmitoyl-phosphatidylcholine
PC	Phosphatidylcholine
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
POPI	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol
PS	Phosphatidylserine
POPS	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
T _M	Phase transition temperatures
DOTAP	<i>N</i> -[1(2,3- dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride
MHF	Micro hydrodynamic focusing
SHF	Single hydrodynamic focusing
DHF	Double hydrodynamic focusing
SRPE	Supercritical reverse phase evaporation
SAS	Supercritical antisolvent
DLS	Dynamic light scattering
SEC	Size exclusion chromatography
NMR	Nuclear magnetic resonance
TEM	Transmission electron microscopy
Cryo-TEM	Cryogenic-TEM
AFM	Atomic force microscopy
EE	Encapsulation efficiency

UV	Ultraviolet
HPLC	High performance liquid chromatography
UPLC	Ultra-performance liquid chromatography
LC-MS	Liquid chromatography–mass spectrometry
SAXS	Small-angle X-ray scattering
DOPC	Dioleoyl phosphatidylcholine
DOPE	Dioleoyl phosphatidylethanolamine
DOPS	Dioleoyl phosphatidylserine
DLPC	Dilauroyl phosphatidylcholine
DMPC	Dimyristoyl phosphatidylcholine
DPPC	Dipalmitoyl phosphatidylcholine
DSPC	Distearoyl phosphatidylcholine
DSPS	Distearoyl phosphatidylserine
DSPE	Distearoyl phosphatidylethanolamine
RES	Reticuloendothelial system
pHLIP	pH Low Insertion Peptide
LTSL	Lysolipids thermosensitive liposomes
LTLD	Lyso-thermosensitive liposomal doxorubicin
RFA	Radiofrequency ablation
HCC	Hepatocellular carcinoma
BBB	Blood-brain barrier
FUS	Focused ultrasound
MLs	Magnetically sensitive liposomes
LSLs	Light-sensitive liposomes
MUC1	Mucin-1
MUC	Mucin
(Boc) ₂ O	Di- <i>tert</i> -butylcarbamate
Boc	<i>Tert</i> -butylcarbamate
CH ₂ Cl ₂	Dichloromethane
CHCl ₃	Chloroform
DIEA	<i>N,N</i> -diisopropylamine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
Et ₃ N	Triethylamine
HCl	Hydrochloric acid
K ₂ CO ₃	Potassium carbonate
KI	Potassium iodide
<i>m</i>	<i>Meta</i>
<i>o</i>	<i>Ortho</i>
<i>p</i>	<i>Para</i>
MeOH	Methanol

MS	Mass spectrometry
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
NaBH ₄	Sodium borohydride
HRP	Horseradish peroxidase
MCF-7	Michigan cancer foundation-7
Vis	Visible
$\lambda_{\max}$	Maximum wavelength
GlcNAc	<i>N</i> -acetylglucosamine
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
CO ₂	Carbon dioxide
RIPA	Radioimmunoprecipitation assay buffer
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
PBS	Phosphate-buffered saline
Neu5Ac	<i>N</i> -acetylneuraminic acid
ARS	Alizarin red s
TX100	Triton X-100
CPPs	Cell penetrating peptides
Rd	Rhodamine
DAPI	4',6-Diamidino-2-phenylindole
ESI	Electrospray ionization
NaH	Sodium hydride
MgSO ₄	Magnesium sulfate
K_{eq}	Equilibrium constant
Gal	Galactose
GalNAc	<i>N</i> -acetylgalactosamine
GlcNAc	<i>N</i> -acetylglucosamine
Ca ²⁺	Calcium ion
DTPA	Diethylenetriamine pentaacetic acid
PET	Photoinduced electron transfer
EPR	Enhanced permeability and retention

1. Introduction to boronic acids and drug delivery vesicles

1.1 Boronic acids: structure, property and application

1.1.1 Structure and property of boronic acids

Synthetically derived from boric acids, boronic acids are trivalent organic compounds with one carbon-based substituent and two hydroxyl groups attached to boron in a planar geometry.[1] Exempt from the octet rule, the sp^2 -hybridized boron atom formally has only six valence electrons, and thus contains an empty p-orbital that renders this group electrophilic. However, this is counteracted by donation of electrons from attached oxygen atoms, resulting in a low energy p-orbital. To be able to exist stably and fulfill the octet rule, boronic acids and their esters may coordinate with basic molecules. To do so, they commonly act as mild organic Lewis acids that accept lone pairs of electrons.

As illustrated in **Figure 1.1**, there are three families of boron-based acids: boric acid, boronic acids and borinic acids, and all can form either neutral or anionic esters, depending on pH, upon reaction with diols through loss of water. Boric acid can form neutral borate esters or anionic borate mono/diesters, while boronic acids can form neutral or anionic boronate monoesters and borinic acids can only form anionic borinate esters upon reaction with diols or divalent ligands.

1.1.2 Complex equilibrium of boronate anion in aqueous environment

The acidic character of the boronic acid group has been an interest among chemists for over a century. Lorand and Edwards reported the first mechanism of polyol complexes of boronic acid and elucidated the structure of the conjugate base of boronic acid (boronate ion).[2] As illustrated in **Figure 1.2**, in aqueous solution, boronic acids in trivalent neutral form are in equilibrium with the anionic tetrahedral form (**Figure 1.2B**). This demonstrates the Lewis acidity of boronic acid because the anionic tetrahedral form of boronate was observed rather than the structure corresponding to a Brønsted base that would result from the reaction shown in **Figure 1.2A**.

The acidity of the boronic acid moiety is characterized by its ability to ionize water and produce hydronium ions, as illustrated in **Figure 1.2B**. Boronic acids with lower pK_a values are equipped with more electrophilic boron atoms that attract nucleophiles, resulting in the formation of anionic tetrahedral hydroxyboronate anions. The pK_a values of boronic acids in aqueous solution are measured by UV spectrophotometry and ^{11}B NMR spectroscopy. Listed in **Table 1.1**, pK_a values of boronic acids have been reported by various research groups.[3-11] In general, arylboronic acids have lower pK_a values than alkylboronic acids. Bulky substituents proximal to the boron atom contribute to higher pK_a values because of the steric hindrance present upon formation of the tetrahedral boronate ion. For example, 2-methoxyphenylboronic acid ($pK_a = 9.0$) is less acidic than 3-methoxyphenylboronic acid ($pK_a = 8.0$).[4, 5] The presence of electron-withdrawing groups on the phenyl ring of arylboronic acids has significant impact on acid strength. For

example, the presence of electron withdrawing ester and nitro substituent drops the pK_a of 3-methoxycarbonyl-5-nitrophenyl to 6.9.[11]

Reported by Wulff and coworkers, the pK_a values of boronic acids can be lowered by neighboring group participation of *ortho*-dialkylaminomethyl substituent.[12] Several mechanisms are proposed to explain the role of anchimeric assistance in acidity of boronic acids.[13-16] It has long been believed that Wulff-type boronic acids undergo intramolecular chelation of amine groups to form B–N bonds. As a result, the hybridization of the boron atom remains tetragonal boronate anion (sp^3) under neutral or slightly acidic conditions, which favors its interaction with diol-containing compounds. However, recently, the Anslyn lab leveraged results from ^{11}B NMR, fluorescence titration and X-ray crystallography experiments to argue that the decrease in pK_a of Wulff-type boronic acids is due to the addition of an electronegative substituent in the aryl ring, such as the protonated ammonium group in this case.[17]

1.1.3 Boronic acid-diol equilibrium in water and protic solvent

The boron–diol interaction plays a critical role in human health, plant growth, and in bacterial quorum sensing.[18, 19] Boron-based compounds can serve as affinity ligands for purification of glycoproteins, nucleotides and carbohydrate transporters, and are also employed as protecting groups for carbohydrates.[20, 21] The first reported boron–diol interaction was discovered by Biot in 1832.[22] His paper reported that the optical rotation of tartaric acid changes following treatment with boric acid. A century later the

interaction between boronic acids and diols were intensely studied. In 1913, Böeseken first reported that the acidity of boric acid increased in the presence of glucose.[23] In 1954, Schultz and coworkers reported the structure of aqueous borate ion by examining its Raman spectrum.[24] In 1959, as shown in **Figure 1.3**, Edwards and co-workers reported quantitative affinities of boric and phenylboronic acids with simple diols and monosaccharides in aqueous solution.[2] The complex equilibrium between model diols and monosaccharides was measured by applying a series of pH depressions. It was concluded that the binding equilibrium is pushed towards the formation of boronate ions when the solution is more basic.

In 2002, Wang and coworkers reported a detailed examination of boronic acid-diol complexation.[25] They found that the pK_a of phenylboronic acid dropped from 8.8 to 6.8 and 4.5 when it formed neutral cyclic borate esters with glucose and fructose, respectively, which indicated that the neutral boronate ester is more acidic than the original boronic acid. Therefore, the boronic acid is less likely to interact with diols when the pH of the solution is relatively low due to the enhanced acidity of the resulting boronate esters. When the pH is elevated, the hybridization of boron shifts from sp^2 to sp^3 , which results in a significant release of angle strain that aids in driving the formation of anionic hydroboronate complexes. To support this theory, Pizer and coworkers reported that stability constants of complexes increase when the aryl substituent on boronic acid is an electron withdrawing group.[3] It is also suggested that the equilibrium constants (K_{eq}) fall into the range of 10^3 - 10^4 for tetracoordinate hydroxyboronate anion

when forming ester with diols and it is at least 10^4 times faster than trigonal neutral boronic acid.[26]

Besides solution pH, the geometry of the hydroxyl groups located in the polyol binding moieties also plays an important role in boronic acid binding interactions. Measured by pH titration and ^{11}B NMR spectroscopy, equilibrium constants of 2.5, 5.5 and 38 were reported for the binding interactions of 1,3-propanediol, 1,2-ethanediol and 1,2,3-propanetriol, respectively, with methylboronic acid.[28] 1,2,3-Propanetriol was much preferred due to the proper cis-diol geometry present in its preferred conformations. The relative affinity of boronates for diols that exist in most carbohydrates is in the order of cis 1,2-diol > cis 1,3-diol >> trans 1,2-diol. Therefore, certain monosaccharides with favored diol geometries have intrinsically higher affinity than others. The association constants of phenyl boronic acid and common diol-containing compounds at pH 7.4 in 10mM phosphate buffer were reported by Wang's lab and are summarized in **Table 1.2**.

1.1.4 Boronic acid-based receptors and sensors for carbohydrates

The chemistry of the boronic acid diol interaction has enabled a broad spectrum of applications. A variety of boronic acid materials can be used as supports for the capture, recognition, and analysis of diol-containing compounds including saccharides, glycoprotein and glycolipids. Examples of such boronic acid materials include microporous monoliths, mesoporous materials, nanoparticles, molecularly imprinted

polymers, polystyryl boronic acid resins, and temperature-responsive materials.[29-36]

One of the most important applications of boronic acid materials is in the enrichment and quantification of glycopeptides and glycoproteins.[37-39]

The Boons lab reported a new strategy for solid-supported oligosaccharide synthesis by using polymeric supported polystyrylboronic acid **1.1 (Figure 1.4)**, where saccharide intermediates can be released and loaded by heat and treatment with solvent mixture.[40, 41]The Lin lab developed a high-throughput method for fabricating a stable and covalent carbohydrate microarray.[42] This approach enabled the study of carbohydrate-protein interactions by immobilizing carbohydrates through boronate ester formation using boronic acids of type **1.2 (Figure 1.4)** fixed on the glass surface and hydroxyl groups of carbohydrates. In 2012, Zhang and co-workers developed a biorthogonal method for modifying magnetic nanoparticles with boronic acid **1.3 (Figure 1.4)** using azide and alkyne click chemistry.[43] In 2013, the Liu lab reported magnetic nanoparticles with dendrimer-assisted **1.4 (Figure 1.4)**, which showed significant enhancement in boronate avidity for the selective enrichment of trace glycoproteins due to the formation of multivalent binding interactions.[44] Besides carbohydrates, boronic acid materials can act as a tool for the identification and purification of other diol-containing compounds, including nucleic acid derivatives containing vicinal cis-diols as well as small chemicals with catechol groups including L-DOPA, catechol estrogens and catecholamines.[45, 46]

1.1.5 Biological and medicinal applications of boronic acids

After the first fluorescent boronic acid-based carbohydrate sensor **1.5 (Figure 1.4)** reported by Yoon and Czarnik in 1992, the ability of boronic acids to form esters reversibly with cis-diols has aroused great interest in the intensive area of sensor and receptor development for monosaccharides, sugar acids, oligosaccharides.[47, 48] For monosaccharides, various boronic acid-based sensors have been developed as blood glucose monitoring devices for diabetes patients.[49] For sugar acids, boron-based sensors have shown great affinity towards tartaric acid, glucuronic acid, and galacturonic acid, as well as sialic acid.[50-54] Focus has since shifted to the development of receptors for complex cell-surface glycoconjugates. The detection of Lewis X, heparin, and Thomsen–Friedenreich (TF) tumor marker by boronic acid-based sensors has been achieved by various research groups.[55-60] In 2002, Wang's lab developed a fluorescent bis-boronate sensor **1.6 (Figure 1.4)** targeting the specific cancer-related oligosaccharide epitope, sialyl Lewis X.[61, 62] The groups of Kataoka and Miyahara have also developed novel methods for quantitative detection of cell-surface sialic acids by immobilized boronic acid-based sensors **1.7 (Figure 1.4)** on gold electrode platforms.[63-65]

An essential aspect to achieve stronger binding interaction with diols when designing boronate affinity material is to lower the intrinsic pK_a . For most biological applications, recognition and detection occurs at pH values around 7.4. Therefore, to obtain higher affinity towards diol-containing compounds, the pK_a of the boronate affinity material should stay close to or even lower than 7.4. Phenylboronic acid (PBA) is one of

the most used boronic acid ligands for developing boronate affinity materials. However, due to its relatively high pK_a values, a basic surrounding pH is usually required for binding interaction. [27]

So far, as summarized by Liu and coworkers, four types of modified boronic acids with intrinsic low pK_a values have been developed.[66] As shown in **Table 1.3**, (I) for boronic acid ligands with electron-withdrawing groups such as nitro, fluoro, sulfonyl and carbonyl, the presence of the EWG on the aryl ring can increase the acidity and lower pK_a values.[35, 67-70] (II) In Wulff-type boronic acids that may contain intramolecular tetracoordinated B–N or B–O bonds, the hybridization of boron atoms remains sp^3 (tetrahedral) for boronate anions under neutral or slightly acidic conditions, which favors its interaction with diol-containing compounds.[12, 71, 72] (III) Benzoboroxoles are viewed as improved Wulff-type boronic acids due to the fact that intramolecular tricoordinated B–O bonds have the same effect as intramolecular tetracoordinated B–N or B–O bonds.[59, 73, 74] Another advantage of benzoboroxole is its excellent hydrophilicity that can be beneficial for biological studies. (IV) Heterocyclic boronic acids with the presence of a heteroatom (N, O, or S) in the ring, which lower the binding pH.[75, 76]

In this dissertation work, we aimed to develop novel boronic acid materials that can recognize carbohydrates, glycoproteins and cell surface glycans, and ultimately serve as agents for cellular drug delivery. To do so, boronic acid moieties were installed on lipid scaffolds to form boronic acid lipids that can be incorporated into liposomes as drug

delivery vesicles. Background information on lipids, liposomes and drug delivery vesicles is discussed in the section below.

1.2 Introduction to drug delivery vesicles

1.2.1 Structure, property and biological roles of lipids

Lipids are biomolecules that are highly soluble in nonpolar organic solvents such as chloroform. Simple lipids such as triacylglycerols or triglycerides are triesters consisting of one glycerol linked to three fatty acids. These simple lipids act as energy storage units among animals. The major reservoir of triacylglycerols resides in the cytoplasm of mammalian adipose cells. The amount of total energy stored in a standard 70 kg man is distributed in 0.17 MJ in glucose, 2.5 MJ in glycogen, 105 MJ in protein and 420 MJ in triacylglycerols.[78] Besides energy reserves, lipids can exist in more complex structures, such as phospholipids, glycolipids, and cholesterol, and act as major building blocks of cell membrane bilayers.[79] Lipid membranes are planar, two-dimensional fluids that separate either cells from the surrounding environment or the cytoplasm of cells from organelles.

Complex lipids can be divided into four main groups: the phospholipids, sterols, sphingolipids and glycolipids, as shown in **Figure 1.5**.[81] Among all, the most important and abundant lipids is the class of phospholipids. Most phospholipids are glycerol-based derivatives and are also named phosphoglycerides. Sterols, such as cholesterol and derivatives, occur naturally in plants, animals, and fungi, and are important components

of biomembranes.[82] They can also function as precursors to various vitamins and hormones. Sphingolipids comprise a complicated class of lipids with a common sphingoid base backbone as a synthetic product of the natural amino acid serine and a long fatty acyl chain CoA.[83] Major sphingolipids include ceramides, which contain an amide-linked fatty acid, phosphosphingolipids, and glycosphingolipids.[84] Glycolipids are lipids with fatty acids attached to a monosaccharide or oligosaccharide via glycosidic linkage. They play an important role in cell membrane stability and serve as recognition sites for cellular interactions.[85] [86]

In this section, the main focus is on the structures and properties of phosphoglycerides that were utilized during the course of this dissertation work. The general structure of phosphoglycerides is shown in **Figure 1.6**. [80] The phosphoglycerides are built up based on one glycerol backbone with two fatty acids that are 12-20 carbon atoms in length esterified to the *sn*-1 and *sn*-2 position of glycerol. The *sn*-3 position of glycerol is where the headgroup is attached, which is in the form of a phosphodiester group in phospholipids. Different classes of phospholipids contain different groups attached onto the phosphodiester moiety of the headgroup. With the hydrophobic fatty acids and hydrophilic phosphate headgroup, phosphoglycerides are amphiphilic. Due to the variation in the specific alcoholic group and fatty acids with different degrees of saturation degree and chain length, a large variety of phosphoglycerides exist in nature. The nomenclature of phosphoglycerides is based on the alcoholic head groups as listed in **Table 1.4**.

1.2.2 Types of delivery vesicles

The concept of drug targeting was first introduced by Paul Ehrlich in the early twentieth century.[87] Ehrlich claimed that in order to increase the accumulation of an active pharmaceutical ingredient (API) in a targeted area of the body, two components were needed – the first one functioned for binding and recognition of the target while the other one functioned as a therapeutic agent for the target.[88] Now, almost a century later, with diligent efforts put forth by scientists around the world, as illustrated in **Figure 1.7**, several pharmaceutical carriers have proven to be efficient for binding and recognition of the targeted cells, tissues and organs in human and animals, including synthetic soluble polymers, microcapsules, microparticles, cells, cell ghosts, liposomes, and micelles.[86, 89, 90] These drug delivery vehicles can minimize drug degradation after intake, prevent undesired side-effects, and increase the bioavailability as well as enrichment of drugs in the pathological area. Besides, they are relatively easy and cheap to make, biodegradable with small particle size, of high loading capacity and exhibit prolonged circulation.[91] Each class of delivery vesicles has its pros and cons, and thus the selection of the most efficient vesicle varies depending on the application.

For synthetic soluble polymers, these can be ideal drug carriers for their desired profile of drug pharmacokinetics, enrichment in the targeted site and drug release. The most widely used polymer materials include *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer and poly(ethylene glycol) (PEG).[92] However, their highly-complex structures and high molecular weights may pose threats to mammalian cells.[93]

Microcapsules and microparticles refer to particles with a size of 1 to 100 μm . These have been established as unique carrier systems for many drugs and tailored to adhere to certain pathological tissue systems.[94] One representative example of microparticle drug delivery systems is to utilize surface modified microparticles with protein shell.[95] Microparticles can enter targeted areas through cross-linking with a second protein or an antibody to its protein shell.[96]

An additional promising delivery system that also happens to be the topic of this dissertation is liposomes. Liposomes are artificial phospholipid bilayer vesicles that can be prepared in various methods to different sizes, components, structures and size distribution. The sizes of liposomes depends on their lipid composition and preparation method and can range from 50nm to greater than 1000nm in diameter.[97] MLVs (multilamellar vesicles) are liposomes with a size of 500 to 5000nm and consist of multiple concentric bilayers. LUVs (large unilamellar vesicles) range from 200 to 800 nm and SUVs (small unilamellar vesicles) are no more than 100nm in diameter. All unilamellar vesicles are formed by a single membrane bilayer.

Micelles represent colloidal dispersions with particle sizes ranging from 5 to 50–100 nm.[98] They can be self-assembled by amphiphilic molecules (lipids) or surface-active agents (surfactants), which consist of two opposite affinities toward either polar or non-polar solvent. Due to their inherent and modifiable properties, micelles are well-suited vesicles for drug delivery purpose.

Liposomes have been considered promising drug delivery vesicles for over two decades since they are biocompatible, low-cost, easy to modify and of quick administration. A liposome injection method has already been on the market for some drug formulations, such as DaunoXome (danorubicin) and Myocet (doxorubicin).[99, 100] Since the liposome is a major topic in this dissertation, more detailed discussions of liposome composition, preparation methods, characterization, classification and liposomal drug delivery can be found in the following sections.

1.2.3 Composition of liposomes

Due to the amphiphilic nature of lipids, they can form a variety of self-assembled structures, such as spherical micelles, cylindrical micelles, spherical bilayers, or planar bilayers.[101] Micelles are single-layered lipid aggregates that have hydrophilic heads facing outwards and hydrophobic tails facing inwards. On the other hand, Bilayers are two-layered sheet with hydrophilic heads pointing both outside and inside, and with hydrophobic tails pointing toward the center of the sheet. Introduced by Israelachvili in 1976, the packing parameter p is used to describe the structural shape of different classes of hydrocarbon amphiphiles that can be used to understand the formation of micelles or bilayers.[102] The equation used to calculate p is $p = \frac{v}{al}$ where v is the volume, a is the cross sectional area of the headgroup, and l is the length of the tail.

As illustrated in **Table 1.5**, when the packing parameter of a certain class of lipids is less than one third, a spherical micelle is formed. An example of a micelle-forming lipid

is lyso-phosphatidylcholine (LPC) with only one acyl chain on the glycerol backbone. As the tail shortens and p is between one third to one half, reversed micelles are formed with the hydrophilic heads facing outwards and hydrophobic tails facing inwards, which then assemble into inverted hexagonal shape.[103] A representative lipid with p between one third to one half is PE. When the tail continues to be shortened to the point that p is between one half and one, a spherical bilayer is formed. Lipids with higher p contribute more to the stability of bilayer membranes while more flexible bilayers are most likely be generated by lipids of p closer to one half. Phosphatidylcholine has a p value close to one and is used as a major component of liposomes due to its ability to form stable planar bilayers.

To form the desired assembly, the choice of lipids is crucial. As mentioned before, PC can self-assemble into spherical bilayer liposomes due to its cylindrical structure. As a result, most liposomes are composed of natural and/or synthetic phospholipids, including phosphatidylcholine as a major component, as well as PE, PS, and PG. Because phospholipids have an intrinsic flip-flop tendency that facilitates leakiness of liposomes, cholesterol can be added to the formulation of liposomes to increase the stability of bilayers.[97, 104-106] Kirby and coworker reported that cholesterol-rich (egg phosphatidylcholine/cholesterol, 7:7 molar ratio) liposomes, when intravenously injected, remained stable in the blood for up to at least 400 minutes regardless of surface charge.[107] Another study has suggested the level of cholesterol can increase the vesicle size.[108]

Besides packing parameter, other properties of lipids are also important to the formation of desired assembly. One example would be the phase transition temperatures (T_M) of phospholipids. Lipid bilayer membranes can exist in either a disordered fluid crystalline state ($T > T_M$), where the hydrocarbon chains are randomly oriented and fluid, or an ordered gel phase ($T < T_M$), where the hydrocarbon chains are fully extended and closely packed, depending on the temperature. Several factors directly affect the phase transition temperature including hydrocarbon length, unsaturation, charge, and headgroup species. Typical phospholipids and their transition temperatures are listed in **Table 1.6**. For drug delivery purposes, phospholipids with proper T_M values remain in the gel state when forming liposomes and encapsulating drugs and change into the fluid state at body temperature. When lipids are in the fluid state, they are more permeable to aqueous solution and drugs can be encapsulated during liposome formation.[109] At body temperatures ($T \approx 37^\circ\text{C}$), a fluid state will make the liposomes leaky, and the encapsulated drugs are likely to be released, ideally at the targeted area.

Charges of component lipids can also greatly affect the property of liposomes.[110] Like lipids, liposomes can have either negative, neutral or positive charges, and liposomes of the same charge are less likely to aggregate due to electrostatic repulsion. Positively charged liposomes such as those containing *N*-[1(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTAP) are commonly used for drug delivery due to several benefits.[111] First, they can increase cell-liposome recognition, interaction and ultimately cellular entry by electrostatic attraction to cell

membranes, which typically are of negative charge.[112] Besides, cationic liposomes are more useful for encapsulating negatively charged compounds such as nucleic acids.

1.2.4 Methods of liposome preparation

The size, lamellarity, and encapsulation efficiency of liposomes can be varied depending on the methods of preparation. Generally speaking, liposomal preparation methods can be categorized as conventional methods and novel methods. The first method for preparing liposomes was reported by Bangham and co-workers in 1976.[113] This method, named after its inventor as the Bangham method or later known as thin lipid film hydration method, involves creating a thin lipid film on a glass surface by evaporating the organic solvents used to dissolve lipids and then a freeze-thaw procedure to guarantee the complete removal of organic solvents. Liposome formation happens after rehydration with aqueous solutions.

Multilamellar vesicles (MLVs) with heterogeneous size distribution are formed with vigorous shaking during rehydration.[114] To obtain vesicles of smaller or more uniform sizes, additional reduction techniques are necessary. For example, small unilamellar vesicles (SUVs) with homogeneous size can be generated with probe sonication. With a titanium probe inserted into the lipid rehydration process, SUVs of desired size can be generated effectively.[115] However, SUVs may be contaminated due to physical contact with metal sonicator probes and phospholipids can be damaged by high temperature exposure. To avoid these risks, most liposomes in this dissertation work

are prepared by using multiple extrusions through a polycarbonate membrane to reduce the sizing.[116] The size of liposomes relies on the number of extrusion cycles and the size of polycarbonate membrane pores. Although not an issue in this dissertation work, low aqueous core entrapment and subsequently low drug encapsulation is a major drawback of this technique.[117]

The conventional techniques for liposome preparation are straightforward for small-scale laboratory setting but are not suitable for large-scale industrial production due to random encapsulation efficiency, batch-to-batch variation, broad size distribution and difficulty in sterilization. With more recent advances in physics and material science, novel methods including micro hydrodynamic focusing (MHF), single hydrodynamic focusing (SHF), double hydrodynamic focusing (DHF), supercritical reverse phase evaporation (SRPE), and supercritical antisolvent (SAS) have been applied to large-scale liposome preparation.[118-121]

1.2.5 Characterization of liposomes

With the development of liposome drug delivery system, several liposomal formulations, such as doxorubicin, daunorubicin, and amphotericin containing liposomes, have successfully reached the market place. To ensure proper liposome performance, batch to batch reproducibility, and stability of the liposome dispersions, Crommelin and Storm have listed several ways to characterize the liposomes, which are discussed in the following section.[122] .

Perhaps the first thing to discuss about liposomes is their size and polydispersity since liposome size is critical for parenteral drug administration, batch to batch quality and variations in manufacture. Methods of liposomal size measurement include dynamic light scattering (DLS), size exclusion chromatography (SEC), nuclear magnetic resonance (NMR), transmission electron microscopy (TEM), cryogenic-TEM (Cryo-TEM), and atomic force microscopy (AFM). The easiest, also most used in this dissertation, technique to measure the liposome size and size distribution is DLS. DLS analyzes the Brownian motion of small particles suspended in aqueous solution resulting in scattering of the incident light. The amount of light scattered is collected and analyzed to give an average size and size distribution of the colliding liposomes. DLS has a wide range of measurement capability (20 – 1000nm) but this technique is sensitive to impurities and cannot differentiate between an individual liposome from liposomal aggregation.[123, 124]

The size of the liposome is an important factor for extravasation and circulation in drug delivery. Liposomes with large diameters (> 400nm) cannot circulate in the blood stream for long since RES uptake can occur in a short period of time after administration.[125, 126] On the other hand, liposomes with short diameters (~ 200nm) can remain in circulation for a long time. The extravasation of liposomes from blood vessels to pathological area is an outcome caused by local blood flow and microvascular permeability.[127] One of the concepts that comes with this is the enhanced permeability and retention (EPR) effect.[128, 129] In order to provide enough nutrition for malignantly spitting cancerous cells, abnormal vessels are found in tumor tissues, in terms of

abundancy, leakiness, metastasis and so on.[130] Liposomes of appropriate size can accumulate relatively easily into tumors tissues through vascular fenestrations and can be retained for a prolonged period time due to the lack of lymphatic drainage.[131] This makes liposomes and other nanoparticles particularly effective for targeting cancerous tissue.

The second aspect related to characterization of liposomes is zeta potential. Liposomes can be negatively or positively charged or uncharged depending on lipid composition and associated ligands. Zeta potential measures the overall charges of liposomes in dispersion and can serve as an index for the stability of the liposomes. Uncharged or low charged liposomes are generally more likely to aggregate over time. In comparison, highly charged liposomes, negatively or positively, are electrostatically repulsive to each other and are less likely to cluster. Zeta potential can be measured by fluctuations in the scattered light using DLS.

Encapsulation efficiency (EE) is another important property of liposome for drug delivery purpose. To obtain a precise measurement of EE, unencapsulated free drug must be removed from suspension by ultracentrifugation, dialysis, or size exclusion chromatography. Next, to release encapsulated drugs, organic solvents, such as acetonitrile, ethanol, and methanol, or surfactants including Triton X-100 can be added to disrupt the integrity of liposome structure. Several techniques can be applied to estimate the abundance of encapsulated content including UV and/or fluorescence

spectroscopy, enzyme- or protein-based assays, gel electrophoresis, HPLC, UPLC, and LC-MS.[132]

Last but not the least, the number of bilayers, in other words lamellarity, is an important parameter in regard to liposomal *in vivo* lifespan and applications. To estimate the number of layers, chemical-based techniques often utilize labeled reagents or radiolabeled ions because these reagents can be uniformly distributed on the surface of outer layer, either to the polar or apolar region of lipid molecules.[133, 134] Other techniques, such as cryo-TEM analysis, ³¹P NMR, small-angle X-ray scattering (SAXS) can also measure liposomal lamellarity.[135-137]

1.2.6 Different types of liposomes

As illustrated in **Figure 1.8**, there are four main types of liposomal drug delivery systems: conventional liposomes, stealth-stabilized liposomes, ligand-targeted liposomes, and a combination of the above.[138] Because of their ability to reduce toxicity compared to free drugs, conventional liposomes are the first generation of liposomes for drug delivery purposes and their clinical application for drug encapsulation, such as doxorubicin and amphotericin, has been studied since the early 1980s.[139-142] Conventional liposomes can be positively or negatively charged or uncharged depending on the presence of either cationic, anionic or neutral phospholipids and cholesterol. The main drawback of conventional liposomes is the short lifetime *in vivo* and quick elimination by the reticuloendothelial system (RES) in mammalian systems.[143]

One aspect that hindered the application of liposomal drug delivery systems in the early days involved the short circulation lifetimes of liposomes. To improve liposome stability and increase their circulation times in the bloodstream, long-circulating liposome (or stealth-stabilized liposomes) that have polyethylene glycol (PEG) chains conjugated on the liposomes surface were introduced that shield liposomes from degradation and therefore enhance accumulation at the targeted pathological area.[144, 145]. The installment of this steric barrier shields liposomal encapsulated agents from opsonization with serum components, and rapid recognition and uptake by RES.[146]

For site-specific delivery of drugs to certain pathological cells, organs or tissues, ligand-targeted liposomal systems have proven to be effective for they can selectively recognize specific ligands that are often over-expressed at the site of the disease.[147] A variety of ligands can serve as targets, such as antibodies, peptides, proteins, carbohydrates, and other small molecules.[148, 149] Since liposomes are self-assembled and dynamic, with proper position of ligands affixed on the liposomal surface, optimal substrate-interaction can be achieved by ligand-targeted liposomes.

In recent years, a newer generation of liposomes, termed theranostic liposomes, has been developed. They combine the merits of the three aforementioned drug delivery platforms in this section and, as a result, can further improve liposomal stability, specific targeting and delivery.[150] Zhan and coworkers reported synthetic ^DCDX-modified stealth liposomes with CDX retro-inverso peptide located on the liposomal surface that can specifically bind to CDX ligand, along with steric stabilization using PEG.[151] This type

of long-circulating immunoliposomes proved to be immunocompatible and significantly enhance drug delivery efficacy.

1.2.7 Liposomal drug delivery

As illustrated in **Figure 1.9**, liposomal drug delivery can generally be categorized into active targeting and triggered-based targeting. Active targeting can be achieved by liposomal surface decoration with target-specific ligands and/or antibodies. On the other hand, triggered-based targeting utilizes internal stimuli, such as pH or enzymes, and external stimuli, such as ultrasound, heat, light, magnetic field.[109]

For active targeting strategies, antibody surface-modified liposomes have been intensively studied for their ability to selectively target diseased cell or organs through the antigen-antibody interaction of specific antibody fragments and monoclonal antibodies affixed on liposomal surface.[152] Munster and coworkers have designed PEGylated doxorubicin liposomes with antibody fragment MM-302 coupled to the outer surface for breast cancer treatment.[153] MM-302 can specifically bind to HER2-overexpressed tumor cells with minimal uptake into healthy cells which express low levels of HER2. This antibody surface-modified liposomal drug delivery system has been under clinical trial to test its toxicity, patient response and clinical benefit.

Since tumor cells have highly variable physiological properties, such as relatively low pH compared to normal cells and over-expression of certain enzymes or glycans, those variations can serve as internal stimuli for liposomal triggered release.[154, 155]

Yao and coworkers have reported a pH-dependent fusion method between liposomes and cellular membranes using pHLP[®] (pH Low Insertion Peptide).[156] pHLP preferentially anchors into lipid bilayers at low pH. The pHLP-modified PEGylated liposomes can enhance membrane fusion, which leads to an increase of cellular uptake, content release and inhibition of cellular proliferation.

For external stimuli-mediated drug delivery, heat, ultrasound, magnetic field, and light are common stimuli for liposomal triggered release. Lysolipids play a major role in thermosensitive liposome for they are structurally different from generally used phospholipids and tend to form micelles on their own.[157, 158] Lipid bilayers of lysolipid thermosensitive liposomes (LTSL) transfer into an unstable gel state when heated and lysolipids can assemble into micelle-like structures and maximize drug release. Tak and coworkers reported lyso-thermosensitive liposomal doxorubicin (LTLD) that can locally release a high concentration of doxorubicin and improve the efficacy of radiofrequency ablation (RFA) for hepatocellular carcinoma (HCC) lesions.[159] Echogenic liposomes serve as great tools for diagnostic imaging and ultrasound mediated drug/DNA delivery. The McDannold lab developed liposomal microbubbles that can permeabilize blood-brain barrier (BBB) when applied to focused ultrasound (FUS).[160] This technique has been investigated in an animal study and can be a noninvasive method for targeted drug delivery in the brain. Magnetically sensitive liposomes (MLs) consist of stabilized iron oxide particles and can be controlled for targeting and drug release by external magnetic field. Nobuto and coworkers reported magnetic doxorubicin liposomes that significantly

suppressed primary tumor growth and enhanced the antitumor effect of systemic chemotherapy compared to standard methods in hamsters inoculated with osteosarcoma.[161] In recent years, light-sensitive liposomes (LSLs) that utilize photosensitizing agents for drug release have been intensively studied. The Best lab has synthesized PC analogue NB-PC with a photocleavable 2-nitrobenzyl group embedded within an acyl chain.[162] Dose-dependent release has been reported with the amount of NB-PC incorporated in PC-based liposomes when the system was exposed to light.

Through the work described in this dissertation, we sought to advance the applications that can be accomplished using boronic acids by incorporating these moieties into complex molecular architectures that can modulate their binding interactions and be exploited to drive chemical and biological processes. To do so, we first developed a boronic acid microplate assay to detect glycoprotein mucin-1 from cancer cell lysates (Chapter two). We then developed boronic acid lipids that consist of a boronic acid headgroup attached to a hydrophobic lipid scaffold that can be incorporated into a liposomal drug delivery platform to drive binding interactions with carbohydrates and glycoproteins and thereby enhance cellular surface glycans recognition and promote cell entry (Chapter three and four). As discussed in the chapters below, by leveraging the merits of both the boron-diol interaction and the intrinsic properties of lipids, our boronic acids materials have proven to be effective agents for targeted drug delivery.

2. A boronic acid assay for the detection of mucin-1 glycoprotein from cancer cells

The scientific data used in this chapter has been published by the authors:

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ChemBioChem. 2017 Aug 17;18(16):1578-1582

2.1. Introduction

2.1.1 Overview

Cell surface glycoproteins are commonly aberrant in disease and act as biomarkers that facilitate diagnostics.[163] Mucin-1 (MUC1) is a prominent example exhibiting truncated glycosylation in cancer. We present a boronic acid microplate assay for sensitive and high-throughput detection of such glycoproteins. Here, the immobilization of biotin-boronic acid **2.1** onto streptavidin plates generates a multivalent surface for glycoprotein recruitment and detection. We first validated the binding properties of **2.1** in solution through titrations with alizarin dye. Next, the microplate assay was explored for HRP analysis as a proof-of-concept glycoprotein with chemiluminescence detection. Finally, this platform was applied for the detection of MUC1 directly from MCF-7 cancer cell lysates using an HRP-tagged antibody that targets the cancerous form of this

glycoprotein. Sensitive dose-dependent detection of MUC1 was observed, showcasing the efficacy of this platform for detecting disease-associated glycoproteins.

2.1.2 Cancer-associated membrane glycoprotein mucin-1

Membrane glycoproteins play critical roles in cell surface binding interactions that control key physiological and pathophysiological processes. Many of these cell surface glycans are aberrantly expressed on diseased cells, and thus act as important biomarkers for disease onset and expression that can be exploited for diagnostic applications.

A prominent example of this phenomenon involves mucin-1 proteins, which contain a transmembrane domain for anchoring to cell membranes as well as an extracellular domain that is heavily glycosylated. Mucin-1 (MUC1), also known as CA15-3, is a type I transmembrane protein with a heavily glycosylated extracellular domain that extends up to 200–500 nm from the cell surface.^[164, 165] Physiologically, the extended negatively charged sugar branches of mucin-1 provide a barrier and anti-adhesive protection to the underlying epithelia from desiccation, pH changes, pollutants and microbes.^[166, 167]

Mucin-1 has two peptide fragments: the long *N*-terminal subunit and the shorter C-terminal subunit associated through stable hydrogen bonding.^[168] Mature functional mucin-1 is heavily *O*-glycosylated and moderately *N*-glycosylated.^[169] It's interesting to note that tumor-associated mucin-1 differs from normal mucin-1, both in its biochemical features and in its cellular distribution. Normally expressed mucin-1 contains heavily

branched core 2 O-glycans, which mainly consist of galactose (Gal) and N-acetylgalactosamine (GalNAc). However, mucin-1 found in breast cancer cells contains mostly the core 1 O-glycans, which mainly consist of Gal, GalNAc and N-acetylglucosamine (GlcNAc).^[170] This change is due to the deactivation of core 2 β 6-GlcNAc-transferase.^[171] Another profound variation of glycosylation is the high level of sialylation in tumor-associated mucin-1, which results in the premature termination of chain elongation and formation of truncated sugar branches.^[172] It is hypothesized that the overexpression of α 2,3- and α 2,6-sialyl-transferase is responsible for increased sialylation.^[173]

Mucin-1 glycoproteins are associated with various forms of cancer including breast, colon, ovarian, lung and pancreatic, and exhibit both abnormal expression and glycosylation.^[171, 174-179] As a matter of fact, truncated glycan chains are seen in cancerous cells that terminate in T and T_n antigens and increased sialic acid. While these variations associated with cancer provide an opportunity for diagnostic purposes, complex glycoproteins are notoriously difficult to analyze using simple assays. Indeed, mucin-1 is difficult to detect using traditional assays including gel electrophoresis and Western Blotting due to its large size, transmembrane properties and varying glycosylation pattern.

2.1.3 Boronic acid sensors for carbohydrates

Boronic acid units have been effective in the development of sensors for carbohydrates through the reversible formation of boronate esters in the binding of diol

moieties on glycans.[180-191] However, individual boronic acid hosts typically exhibit low binding affinities towards carbohydrates in aqueous solution due to solvent competition. This can be overcome through the use of multivalent boronic acid-presenting surfaces, in which the synergistic effect of the binding of multiple glycans to binding groups can dramatically enhance the binding affinity.[192-199] Boronic acids exhibit varying affinities for different sugar units depending upon charge and diol orientation, and indeed the sialic acid moiety of cancer-associated mucin-1 is among those that bind with the highest affinity.

2.1.4 Microplate assays

Microplate and microarray assays provide an effective means for convenient and high-throughput detection of biomolecules directly from complex biological samples.[200-212] These platforms rely upon the ability to recruit biological molecules to surface-immobilized ligands, followed by the detection of bound proteins. Often, antibody-based detection is exploited, which benefits from the strong affinity and selectivity of these reagents for particular proteins, and indeed specific forms of the protein associated with the disease (ie sequence, posttranslational modification, etc.). Finally, antibody-based utilizing chemiluminescence signal transduction benefits from high sensitivity and convenience. For these reasons, we set out to develop a boronic acid-based microplate assay for glycoproteins, with mucin-1 as a significant initial target. We envisioned that the immobilization of boronic acid moieties onto microplate surfaces

would produce a dense clustering that would result in high affinity binding interactions with glycoproteins,[196, 197] and that chemiluminescence-based signaling would enable the sensitive detection of target disease-related proteins.

In order to develop a high-throughput assay for the detection of the cancer-associated glycoprotein mucin-1, we first set out to adapt the boronic acid sensor platform for use within the context of a surface-based assay. For this purpose, a strategy for the immobilization of the boronic acid sensing unit was needed. We and others have previously exploited streptavidin-coated microplates and microarray slides as beneficial surfaces for the non-covalent immobilization of binding moieties through the strong non-covalent streptavidin–biotin interaction in a manner that limits non-specific binding interactions.[213-216] Thus, we elected to pursue a boronic acid–biotin conjugate for the deposition of the sensing unit onto these surfaces.

2.2 Design and synthesis of biotin-boronic acid conjugate

The design and synthesis of boronic acid–biotin **2.1** used in this study is depicted in **Figure 2.1**. This compound contains a biotin moiety for immobilization onto streptavidin-coated surfaces as well as a boronic acid for the capture of glycan-containing proteins and molecules. An ethylene glycol tether was introduced between these two units to space the binding group from the derivatized surface. An *ortho*-(alkylaminomethyl)phenylboronic acid unit was incorporated since the phenylboronic

acid unit is known to stabilize boronic acid group and deter oxidation while the aminomethyl group has been used to enhance carbohydrate binding affinity, particularly at lower pH closer to physiological conditions.[217, 218] In the synthesis of **2.1**, phenylboronic acid **2.2** was first coupled to mono-Boc protected linker **2.3**, which was synthesized from the corresponding diamine precursor, through a reductive amination. The remaining free amine of the product, **2.4**, was then coupled to biotin succinimidyl ester **2.5** to yield **2.1**.

2.3. Initial validation of boronic acid binding properties with alizarin dye

Following the synthesis of **2.1**, we next set out to analyze binding interactions with this molecule through titration with the dye alizarin, which undergoes a shift in photophysical properties upon binding to boronic acids through its catechol moiety.[219] To do so, we first performed UV/Vis absorbance experiments in which we titrated a solution of the dye with boronic acid **2.1** and observed the expected shift in absorbance properties, with the λ_{max} increasing from ~ 430 nm to 470 nm. This is shown in the form of both a spectral overlay (**Figure 2.2A**) and a plot of intensity at 470 nm (**Figure 2.2B**). Additionally, a fluorescence titration was performed through excitation at 450 nm. This also led to the expected increase of fluorescence at ~ 600 nm, as indicated by spectral overlay (**Figure 2.2C**) and a plot of emission at 592 nm (**Figure 2.2D**). Collectively, these

titrations show that compound **2.1** binds to alizarin red S as expected for a boronic acid sensor.

2.4 Boronic acid microplate assay for mucin detection

We next moved to implement boronic acid–biotin conjugate **2.1** for a surface-based assay for glycoproteins as depicted in **Figure 2.3**. Here, the biotin moiety is exploited to immobilize this molecule onto the surface of streptavidin-coated microplates. This creates a high density boronic acid surface so as to enhance the binding affinity towards glycoproteins through the formation of multivalent binding interactions.[202, 220-223] This is critical for the formation of complexes with sufficient stability for detection purposes, particularly since individual boronic acids exhibit relatively low affinities for monosaccharides. We first chose to test this platform for the detection horseradish peroxidase (HRP), which is commonly used as a test case for the detection of glycoproteins. HRP has been found to contain significant carbohydrate composition including *N*-acetylglucosamine (GlcNAc), mannose, fucose and xylose units composing the complex oligosaccharides.[224, 225] The choice of HRP also facilitates signal transduction, as the protein can be used to catalyze the excitation of luminol, resulting in chemiluminescence indicating the presence of protein bound to the surface. Thus, these

initial studies do not require the HRP-tagged antibody depicted in **Figure 2.3** that is necessary for the detection of mucin-1.

For HRP studies, separate wells of 96-well streptavidin-coated microplates were coated with uniform solutions containing 200 nM concentrations of boronic acid–biotin conjugate **2.1** and washed to remove unbound molecules. Next, the functionalized surface was treated with solutions of HRP of varying concentrations ranging from 0 - 100 μ M, and again washed to remove unbound protein. Finally, the presence of bound protein was evaluated using the Pierce supersignal ELISA femto maximum sensitivity substrate kit. As can be seen in **Figure 2.4A**, a dose-dependent response in chemiluminescence was observed relative to the amount of treated protein. Negative controls in which wells were not treated with **2.1**, but the assay was otherwise run in the same manner, yield essentially no signal (data not shown). These results indicate the efficacy of this platform for the binding and detection of glycoproteins.

Following this proof-of-concept, we set out to apply the boronic acid surface assay to the detection of cancer-associated mucin-1. Purified mucin-1 protein is difficult to access in a form in which glycosylation patterns are understood. Therefore, we turned our attention to the detection of mucin-1 from cancer cell lysates, which was also preferable for performing the analysis in a complex biological environment towards diagnostic applications. For this, MCF-7 breast cancer cells were selected due to elevated presentation of mucin-1. The assay was performed as previously described and illustrated

in **Figure 2.3**, except microplates were treated with varying concentrations of MCF-7 lysate, followed by treatment with an HRP-tagged anti-MUC-1 SM3 antibody, with washes in between each step. This antibody is designed to target the under-glycosylated form of mucin-1 found in cancer cells and shows minimal binding affinity towards normal mucin-1 glycoproteins. As can be seen in the chemiluminescence results shown in **Figure 2.4B**, we once again obtained a dose-dependent response to treated cell lysate. We additionally ran a negative control in this study in which wells were not treated with **2.1**, but the assay remained the same otherwise. These wells led to no signal, as shown in **Figure 2.4B**, demonstrating that the boronic acid sensor **2.1** is needed for glycoprotein recruitment and detection. This validates the detection of cancer-associated mucin-1 directly from cancer cells using this platform.

2.5 Summary and future outlook

The described boronic acid assay provides a convenient and promising platform for the rapid detection of disease-associated glycoproteins directly from biological samples. The surface immobilization of the boronic acid binding moiety provides a multivalent binding surface designed to increase the affinity towards complex glycoproteins, thereby enabling a wash-off detection assay. The results show that both purified proteins (HRP) and proteins within cell lysates (mucin-1) can be bound and detected. The assay is expected to be generalizable based on the availability of antibodies

against different disease-associated glycoproteins, and since this signal transduction method can be used in conjunction with antibodies that bind to specific forms of the protein (sequence, glycosylation, etc.) associated with disease states. Overall, this assay overcomes significant challenges associated with the detection and characterization of complex glycoproteins, and provides a promising means for analyzing these complex biomolecules in a manner that is amenable to diagnostic application.

2.6 Material and method

General. Reagents and solvents were generally purchased from Acros, Aldrich or Fisher Scientific and used as received. *tert*-Butyl (3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate (**2.3**)[226] and biotin-succinimidyl ester (**2.5**)[227] were synthesized as described previously. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies and C18 (17%) reverse phase SPE columns (6ml, 2g) from Silicycle. NMR spectra were obtained using Varian Mercury 300 or 500 MHz spectrometers. Mass spectra were obtained with JEOL DART-AccuTOF or Applied Biosystems/ QStar Elite HPLC—QTOF spectrometers. UV-vis absorbance titration experiments were performed using an Evolution 600 UV-vis instrument from ThermoFisher Scientific. Fluorescence titration experiments were performed using a Cary Eclipse Fluorescence

Spectrophotometer from Agilent Technologies. Chemiluminescence and fluorescence microplate studies were performed using a Synergy 2 Multi-Detection Microplate Reader from BioTek Instruments, Inc. Pierce Streptavidin Coated 96-Well Plates and SuperSignal Chemiluminescent Substrates were purchased from ThermoFisher Scientific. Type VI Peroxidase from horseradish was purchased from SigmaAldrich. Mouse monoclonal MUC-1 Antibody (SM3) tagged HRP-conjugate was purchased from Novus Biologicals. Water for binding studies was purified using a Milli-Q filtration system. Data were plotted and curve-fitted using SigmaPlot. All plots represent the average of at least 3 experiments with error bars included +/- the standard error for each set of measurements.

Synthesis

(2-(19,19-dimethyl-17-oxo-6,9,12,18-tetraoxa-2,16-diazaicosyl)phenyl)boronic acid (2.4)

2-Formylphenylboronic acid (**2.2**, 0.258 g, 1.72 mmol) was dissolved in 20 mL of a mixture of ethanol/toluene (90:10) and then amine **2.3** (0.5 g, 1.56 mmol) was added. A Dean-Stark trap was fixed to the reaction vessel and filled with 10 mL of the same solvent mixture. The reaction was then allowed to stir for 16 h at 100 °C. Next, the reaction was cooled using an ice bath. Sodium borohydride (0.118g, 3.12mmol) was then added slowly, after which the reaction mixture was allowed to stir for 2 h at 0 °C. The solvent was next removed through rotary evaporation and the resulting crude was purified using C18(17%) reverse phase solid phase extraction (SPE) column chromatography with a gradient

water/methanol solvent system. Compound **2.4** was obtained as a pale yellow gel (0.254 g, 0.56 mmol, 36% yield).

¹H NMR (300MHz, MeOD): δ 1.42 (s, 9H), 1.66-1.74 (p, 2H J=6Hz), 1.74-1.83 (p, 2H, J=9Hz), 2.65-2.69 (t, 2H, J=6Hz), 3.08-3.13 (t, 2H, J=9Hz), 3.31-3.34 (t, 2H, J=6Hz), 3.46-3.60 (m, 12H) 3.73 (s, 2H), 7.29-7.33(m, 4H) DART-MS [M+H]⁺: calcd for C₂₂H₄₀BN₂O₇ 455.2929.

(2-(17-oxo-21-((3a*R*,4*R*,6a*S*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-6,9,12-trioxa-2,16-diazahenicosyl)phenyl)boronic acid (2.1)

Amberlyst® 15 resin (2.5 g) was soaked in methanol overnight. Next, this resin was neutralized by soaking in 20 mL ammonia saturated methanol solution for 24h, followed by a wash with 75mL 3M hydrochloric acid in 50% methanol solution. Finally, the resin was washed with 25mL methanol, 25mL tetrahydrofuran and 25mL dichloromethane. Compound **2.4** (0.1g, 0.22mmol) was dissolved in 5mL of anhydrous methanol, followed by the addition of the cleaned Amberlyst® 15 (0.5g). The reaction was allowed to stir overnight. Next, the reaction mixture was filtered and the solvent was removed by rotatory evaporation. Purification of the deprotected amine was performed using C18(17%) reverse phase SPE columns with a gradient water/methanol solvent system, which yielded a pale yellow oil (0.070 g) that was passed onto the next step without further purification. This was dissolved in 2 mL dry DMF and distilled triethylamine (Et₃N, 0.03 mL, 0.22 mmol) and biotin succinimidyl ester (**2.5**, 0.075 g, 0.22 mmol) were added. The reaction was then allowed to stir overnight at rt, after which the solvent was removed

by rotatory evaporation. Compound **2.1** was purified using C18(17%) reverse phase SPE column chromatography with a water/methanol gradient solvent system. The product obtained as pale yellow oil (0.024 g, 21% yield).

^1H NMR (500MHz, MeOD): δ 0.87-0.97 (m, 1H), 1.28 (s 2H), 1.37-1.48(t, 3H, $J=15\text{Hz}$), 1.55-1.76(m, 6H), 1.96-2.04 (p, 10H), 2.16-2.21 (t, 2H, $J=15\text{Hz}$), 2.64-2.67 (m, 1H), 2.71-2.72 (m, 1H), 2.89-2.93 (dd, 1H, $J=5\text{Hz}$), 3.00-3.05 (t, 2H, $J=10\text{Hz}$), 3.16-3.28 (m, 3H), 3.38-3.50 (m, 5H), 3.60 (s, 3H), 3.63-3.67 (t, 2H, $J=10\text{Hz}$), 4.04(s, 2H), 4.27-4.32 (m,1H), 4.46-4.50 (m, 1H), 7.13-7.21 (m, 2H), 7.42-7.44 (m, 2H). ^{13}C NMR (500MHz, MeOD): δ 25.47, 26.07, 28.10, 28.39, 28.72, 29.02, 31.65, 35.42, 36.39, 39.62, 46.19, 48.43, 53.57, 55.59, 60.20, 61.96, 68.45, 69.75, 69.86, 69.99, 70.05, 70.09, 126.24, 126.92, 164.66, 174.49. ESI-MS $[\text{M}+\text{H}]^+$: calcd for $\text{C}_{27}\text{H}_{46}\text{BN}_4\text{O}_7\text{S}$ 581.318, $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$: calcd for $\text{C}_{27}\text{H}_{44}\text{BN}_4\text{O}_6\text{S}$ 563.3074 found 581.2982, 563.2924 $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$.

Fluorescence and absorbance binding studies of boronic acid and ARS.

2 mL of a 100 μM solution of alizarin in methanol was added to an empty cuvette. This was titrated with a solution containing alizarin (100 μM) and biotinylated boronic acid **2.1** (4.1 mM), which was added in 10 μL increments until the concentration of boronic acid was 5 times as much as the concentration of alizarin. Absorbance and fluorescence intensities were measured after each titration. Absorbance intensities were scanned from 200 to 700 nm. The excitation wavelength for fluorescence titrations was set at 450 nm and fluorescence intensities were measured from 500 to 700nm. Each study was repeated

at least three times.

Microplate HRP binding studies.

Peroxidase from horseradish Type VI was purchased from Sigma Aldrich ≥ 250 units/mL (U/mL). A stock solution of this protein of ~ 100 U/mL was prepared in wash buffer. The wash buffer used in this study consisted of 10mM PBS at pH 8.0 with 0.01% Tween 10. Experiments were performed in black 96-well microplates. First, all wells to be used were incubated with 200 μ L of the wash buffer for 30 min. After removing the wash buffer, to each well was added 100 μ L of a 200 nM solution of boronic acid–biotin **2.1** in wash buffer. Solutions were incubated for 1 h, removed, and the wells were washed three times with 250 μ L wash buffer for 5 min each. Next, solutions of varying concentrations of horseradish peroxidase (0, 1, 2, 4, 8, 10, 15, 20, 40, 80, and 100 U/mL) in wash buffer were added into separate wells. These solutions were incubated for 1 h, removed, and the wells were washed three times with 250 μ L of wash buffer for 5 min. Chemiluminescence detection of surface-bound HRP was next performed using supersignal ELISA femto maximum sensitivity substrate. For detection, a 1:1 mixture of the substrate and peroxide solutions was prepared, and 100 μ L of the resulting solution was added to each well and mixed with a multichannel pipetter. The microplate was then immediately placed in a microplate reader, and chemiluminescence was repeatedly measured using a 440nm with a bandpass of 20 nm filter for 10 min. Data were plotted and curve-fitted using SigmaPlot.

Plots represent the average of at least 3 experiments with error bars included +/- the standard error for each set of measurements.

MCF-7 cell lysate preparation

Human breast cancer MCF-7 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM). Culture media was supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin, and culture cells were maintained at 37°C under a humidified atmosphere of 5% CO₂. For the cell lysate, MCF-7 cells were grown to 60- 80% confluence in 6-cm plates. Total cell lysates were then isolated using RIPA buffer (1×PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (1 mM PMSF, 5 µg/mL aprotinin and 5 µg/mL leupeptin) and phosphatase inhibitors (1 mM Na₃VO₄ and 1 mM NaF). The cell was sonicated 3 times. Protein concentration was determined to be 0.148 mg/mL by the BCA protein assay (Pierce, Rockford, IL), using BSA as the standard.

Microplate binding studies of MUC1 in MCF-7 cell lysate.

The wash buffer used in this study consisted of 10 mM PBS at pH 8.0 with 0.05% Tween 10. Black streptavidin- coated 96-well microplate wells were incubated with 200 µL of the wash buffer for 30 min. After removal of wash buffer, 100 µL of a 200 nM solution of

biotin-boronic acid **2.1** in water was added into different rows. A negative control was also run in which wells were not treated with boronic acid **2.1**, but otherwise experiments were run the same. After 1 h of incubation, these solutions were removed, and the wells were washed three times with 250 μ L wash buffer for 5 min. MCF-7 cell lysate (0.148 mg/mL) was diluted 50-fold into wash buffer to produce a stock solution of 2.96 μ g/mL. Next, aliquots of this stock lysate (0, 1, 3, 5, 6, 7, 8, 9, 10, 12, 15, and 20 μ L) were diluted into wash buffer to a total volume of 100 μ L, producing solutions of 0, 29.6, 88.8, 148, 178, 207, 237, 266, 296, 355, 444, and 592 ng/mL. These solutions were added to separate wells of the microplate functionalized with **2.1**. After 1 h of incubation, these solutions were removed, and the wells were washed with 250 μ L wash buffer three times for 5 min each. HRP- tagged MUC1 antibody was diluted with wash buffer in a 1:500 ratio according to manufacture instructions (from 0.1 mg/mL to 200 ng/mL). Then, 100 μ L of the resulting solution was added to each well. After 1 h of incubation, these solutions were removed, and the wells were washed three times with 250 μ L for 5 min each. Finally, chemiluminescence detection of the HRP anti-MUC1 antibody was next performed using supersignal ELISA femto maximum sensitivity substrate. For detection, a 1:1 mixture of the substrate and peroxide solutions was prepared, 100 μ L of the resulting solution was added to each well, and solutions were mixed using a multichannel pipetter. The microplate was then immediately placed in a microplate reader, and the chemiluminescence was repeatedly measured using a 440nm with a bandpass of 20nm filter for 10 min.

3. Boronic acid liposomes for cellular delivery and content release driven by carbohydrate

The scientific data used in this chapter has been published by the authors:

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Barrera and Michael D. Best. Chem. Commun., 2018, 54, 6169

3.1 Introduction

Liposomes have emerged as sophisticated carriers for delivering therapeutic cargo.[71] Indeed, liposome-based drug delivery platforms have been approved by the FDA and many more are in the late-stage of clinical trials.[228] To advance liposomal drug delivery, two important areas of research include the enhancement of cell infiltration by liposomes, and the ability to control cargo delivery by triggering the release of contents. For the former, unnatural cationic lipids such as 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) or lipids conjugated to cell penetrating peptides (CPPs), such as octaarginine (R8), are commonly used to infiltrate membranes.[229, 230] However, these cationic lipids commonly exhibit significant toxicity.[231] Regarding triggered release, approaches exploiting both active and passive stimuli, such as light, redox, pH, enzymes and temperature have been explored, but there are drawbacks in each case.[109, 232, 233] Drawbacks of passive release include the minimal variations that are exploited for

selective release, such as the slight increase in acidity in cancer cells (pH ~ 6.5-6.9) compared to healthy cells (pH ~ 7.2-7.4).[234, 235] Active release protocols are hindered by challenges in delivering external stimuli, such as poor tissue penetration using light-induced release. Herein, we report boronic acid liposomes as a means for enhancing both cell infiltration and content delivery based on carbohydrate binding interactions.

Aberrant glycosylation patterns, both in terms of carbohydrate composition and abundance,[236, 237] are linked with diseases such as oncogenic transformation. For example, glycosyltransferase dysregulation leads to increased sialylation of truncated gangliosides and overexpressed complex β -1,6-branched *N*-linked glycans on human melanoma cells.[238, 239] Such cell-type specific complex glycan alternations can provide a handle for selective cell targeting and delivery. The boronic acid molecular recognition unit has been extensively used to bind and separate carbohydrates through reversible formation of boronate esters.[183, 188, 190] However, biological application of this sensing group is challenged by the relatively low binding affinity in aqueous media. This can be overcome through multivalent binding interactions, in which avidity effects lead to exponential enhancements in affinity.[194] Smith and co-workers have previously shown that boronic acid lipids are effective at driving calcium-dependent liposome fusion[240] and at enhancing the binding of cell membranes.[241] In this chapter, we delve into the efficacy of boronic acid liposomes as a means to enhance both cellular infiltration and targeted content release driven by carbohydrate binding.

3.2 Design and synthesis of single-tailed and dual-tailed boronic acid

This project began with the design of boronic acid lipid conjugates to present this recognition group on the surface of resulting liposomes. One such compound is lipid **3.1**, in which the boronic acid is directly attached onto an aminoglycerolipid scaffold. An ortho-(alkylaminomethyl)phenylboronic acid binding unit was chosen since the phenyl group is known to stabilize the boronic acid while the amino moiety enhances carbohydrate binding affinity at physiological conditions.[217, 218] The synthesis of **3.1**, shown in **Figure 3.1**, began with racemic 3-aminopropane-1,2-diol (**3.2**), the amine of which was first protected as a phthalimide in **3.3**. Next, a Williamson ether synthesis was used to introduce hydrophobic alkyl chains using. Ether-linked lipid chains were employed to circumvent potential hydrolysis by lipase enzymes *in vivo*. The phthalimide was next deprotected to produce **3.4**, which was followed by a reductive amination reaction to produce **3.1**. We additionally designed, synthesized and studied single-chain boronic acid lipid **3.5** analogous to a fatty acid (**Figure 3.2**). This alternative lipid exhibited similar properties as **3.1** during release studies, with results reported in the sections below.

3.3 Fluorescence-based dye leakage assay

We first evaluated triggered release from liposomes containing **3.1** or **3.5** upon treatment with the polysaccharide heparin as a model carbohydrate. Heparin

is an anticoagulant consisting of repeating disaccharide units of sulphated iduronic acid/glucuronic acid and glucosamine residues, and has previously been shown to bind to boronic acids in a multivalent manner.[242, 243] Additionally, heparin sulfate proteoglycans have been implicated for driving the cellular entry of cationic liposomes through binding interactions.[244] We initially examined the release of hydrophobic contents from liposomes using a Nile red release assay, in which liposomal solubilization of this insoluble dye gives way to fluorescence decreases when the dye is released into aqueous solution and precipitates.[245, 246] A cartoon depicting the release of both hydrophobic and hydrophilic contents is shown in **Figure 3.3**.

Unilamellar liposomes composed of 0% to 25% of boronic acid lipid **3.1** doped into L- α -phosphatidylcholine (PC, mixed isomers) were first prepared with Nile red included in the formulation. Hydration, freeze-thaw, sonication and extrusion through 200 nm membranes were performed to generate liposomes with uniform size. As seen in **Figure 3.4A**, titration of Nile red-loaded liposomes with heparin led to a decrease in fluorescence in a manner that was dependent on the percentage of boronic acid lipid **3.1** incorporated within the liposomes. Specifically, control liposomes containing 0% of **3.1** showed minimal background release of Nile red (~5% fluorescence decrease), while this decrease was accentuated with increasing boronic acid lipids (5% **3.1**, ~10% decrease; 10% **3.1**, ~25%; and 20% **3.1**, ~50%). The extent of release appeared to reach a plateau since

20% and 25% of **3.1** in liposomes yielded similar results. Control Nile red release and dynamic light scattering (DLS) studies in which compound **3.4** was instead included within liposomes showed minimal change, indicating the necessity of the boronic acid moiety of **3.1** (**Figure 3.5**). In addition, titration with horseradish peroxidase (HRP) glycoprotein as an alternative model with different carbohydrate composition[224, 225] also yielded greater release using liposomes containing **3.1** (**Figure 3.6**), indicating generality and ruling out simple electrostatic binding. These data provide evidence that the boronic acid units presented on the liposomes bind to carbohydrates, thereby triggering Nile red release. In addition, the threshold-type response we observed suggests the formation of multivalent binding interactions that we expect to play a key role in binding and release.[202]

We anticipated that liposome release would be driven by lipid reorganization upon carbohydrate binding in a manner that perturbs membrane bilayer packing to stimulate content release. To assess this hypothesis, DLS experiments were conducted to probe for changes in the particle sizes of lipid assemblies before and after heparin treatment (**Figure 3.4B**). These are reported using an exponential scale. Here, the inclusion of **3.1** showed no effect on initial liposome size. Upon treatment with heparin, liposomes containing 0 or 5% **3.1** exhibited minimal changes in size, which matches the slight decrease in Nile red fluorescence observed during release studies. Significant increases in particle sizes were detected following heparin addition to liposomes containing 10-20% of **3.1**.

Finally, DLS studies of PC liposomes containing either 0% or 10% of **3.1** also showed no significant changes over the course of a week at room temperature (data not shown), indicating that these liposomes are robust.

We next studied carbohydrate-induced release of the polar fluorescent dye sulforhodamine B.[247] In this assay, the dye is encapsulated within the liposome aqueous core at high concentrations such that it is quenched, and size exclusion chromatography (SEC) is used to remove unencapsulated fluorophore. Dye release then leads to an increase in fluorescence intensity in bulk solution (**Figure 3.3**). The extent of dye release in this assay is often variable based on the amount of soluble dye that is entrapped when liposomes form and the variability of liposome concentrations obtained by SEC purification. Thus, we normalized dye release as a percentage of total release caused by final treatment of the liposomes with the detergent Triton X-100. This assay culminated in dose-dependent increases of sulforhodamine B fluorescence based on the percentage of **3.1** incorporated in the liposomes (**Figure 3.7**). These results indicate that boronic acid **3.1** is also effective for triggered release of polar contents from liposomes, which is more challenging as this requires that polar/charged molecules escape the hydrophobic membrane barrier. Boronic acid lipids **3.1** and **3.5** exhibited very similar properties in both the Nile red and sulforhodamine release studies, and thus the results for **3.5** are shown in **Figures 3.8** and **3.9**. Similarities in these data suggest that the particular lipid scaffold that anchors the boronic acid in the membrane is not critical for activity.

Both lipids do possess *ortho*- (alkylaminomethyl)phenylboronic acid moieties known to enhance binding affinity at physiological pH.[217, 218]

3.4 Scanning transmission electron microscopy studies

To further explore the issue of changes in assembly, scanning transmission electron microscope (STEM) images were taken before and after heparin treatment. Here, standard liposome images were observed for samples containing 10% of **3.1** initially, while heparin treatment resulted in much larger and complex membrane assemblies (**Figure 3.10**). On the other hand, images of control PC-only liposomes treated with heparin showed liposomes with sizes similar to untreated samples. The DLS and STEM data provide evidence for lipid reorganization driven by heparin binding only when **3.1** is present, which could be explained by carbohydrate-promoted processes such as fusion, aggregation and/or the formation of different supramolecular lipid assemblies.

3.5 Microplate assay to confirm carbohydrate binding

We next sought to verify that boronic acid liposomes bind to carbohydrates to further justify that release may be caused by molecular recognition. A microplate assay was used to assess the binding of fluorescent liposomes to a commercially available heparin–biotin conjugate immobilized onto streptavidin-

coated microplates. We have previously used similar assays to study liposome and lipid binding interactions.[248-250] Liposomes in this study contained either 0% (control) or 20% of **3.1**, 1% rhodamine-labeled phosphatidylethanolamine (Rd-PE) as a fluorescent marker, with the remainder PC. The results (**Figure 3.11**) indicate a significant enhancement in surface binding when **3.1** was present in the liposomes, thereby validating the heparin-binding ability of liposomes containing **3.1**. In an additional control, liposomes containing **3.1** yielded minimal signal when heparin-biotin was not added (data not shown).

3.6 Cellular delivery studies of boronic acid liposomes

We next assessed the ability of lipid **3.1** to promote liposome cell entry. Boronic acid liposomes were expected to bind to cell surface carbohydrates and enhance proximity to plasma membranes, thereby boosting cell entry pathways such as membrane fusion or pinocytosis (**Figure 3.12A**). Confocal fluorescence laser scanning microscopy experiments were used to evaluate the delivery of liposomes containing 0.08% of Rd-PE. Control liposomes with only PC and Rd-PE were compared to study samples doped with 10% of **3.1**. Liposomes were incubated with A375 melanoma cells for one hour at 37°C, washed to remove residual free liposomes, labelled with DAPI, fixed and mounted on glass slides for imaging. Representative images are shown in **Figure 3.12**. Cells treated with liposomes containing 10% of **3.1** yielded a dramatic enhancement in fluorescence

(**Figure 3.12C**) compared to control liposomes lacking **3.1** (**Figure 3.12B**). These results show that **3.1** is effective for driving liposomal cell entry. As an initial assessment of toxicity, cells treated with liposomes containing 10% **3.1** and 0.08% Rd-PE were observed to be healthy after incubation, while liposomes containing 15-20% of **3.1** with 0.08% Rd-PE led to observable toxicity effects including some cytoplasmic shrinking and nuclear distortion, particularly at longer incubation times, which may result from membrane disruption. While extensive studies are required to assess the clinical viability of this platform, these results showcase boronic acid lipid **3.1** as a promising agent for cellular delivery.

3.7 Summary and future outlook

In conclusion, we have found that liposomes containing boronic acid lipid **3.1** are effective for content release and cell entry driven by carbohydrate binding. These liposomes could provide versatility by either delivering therapeutic cargo directly via cell entry or by releasing contents upon in close proximity to cells, both of which are likely to enhance delivery. While this provides a promising means for general cellular delivery applications, it also opens up the possibility of selective delivery to diseased cells based on the specific composition and abundance of cell surface carbohydrates. In particular, boronic acid dimers have been reported that exhibit selective binding to tumor markers such as sialyl Lewis X.[251] Future work will be aimed at studying and enhancing cell type-specific cellular delivery using

boronic acid moieties similar to these adapted for presentation on liposome surfaces.

3.8 Materials and method

General experimental. Reagents and solvents were generally purchased from Acros, Aldrich or Fisher Scientific and used as received. Heparin lithium salt from porcine intestinal mucosa was purchased from Sigma-Aldrich, Inc. L- α -Phosphatidylcholine (mixed isomers) and L- α -phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) were purchased from Avanti Polar Lipids, Inc. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using Varian Mercury 300 or 500 MHz spectrometers. Mass spectra were obtained with JEOL DART-AccuTOF or Applied Biosystems/ QStar Elite HPLC—QTOF spectrometers. Fluorescence titration experiments were performed using a Cary Eclipse Fluorescence Spectrophotometer from Agilent Technologies. Water for liposome preparation and trigger-release studies was purified using a Milli-Q filtration system. Data were plotted and curve-fitted using SigmaPlot 13. Dynamic Light scattering measurements were performed to study the size and stability of liposomes using a Malvern Zetasizer Nano ZS instrument equipped with a He-Ne 633 nm laser and a temperature controller at a scattering angle of 173°. All plots represent the

average of at least 3 experiments with error bars denoting +/- the standard error for each set of measurements.

Synthesis

(2-((dodecylamino)methyl)phenyl)boronic acid **3.5**

2-Formylphenylboronic acid (0.89 g, 5.929 mmol) and triethylamine (1.09 g, 10.78 mmol) were dissolved in 10 mL of a mixture of ethanol/ toluene (90:10) in a 50 mL round-bottomed flask and then dodecan-1-amine (1 g, 5.39 mmol) was added. A Dean-Stark trap was fixed to the reaction vessel and filled with 10 mL of the same solvent mixture. The reaction was then allowed to stir for 16 h at 100°C, and then cooled using an ice bath. Sodium borohydride (0.82 g, 21.56 mmol) was then added slowly, after which the reaction mixture was allowed to stir for 2 h at 0°C. The solvent was next removed through rotary evaporation and the resulting crude was purified through silica gel column chromatography with an increasing gradient of ethyl acetate/ hexane solvent system from 25% to 100% and then flushed with 100% methanol. The product was then dissolved in dichloromethane and filtered. Then, dichloromethane was removed under reduced pressure to yield compound **3.5** as a white gel (1.29 g, 4.03 mmol, 85% yield). ¹H NMR (300 MHz, Chloroform-d) δ 7.53 – 7.41 (m, 2H), 7.35 – 7.28 (m, 2H), 3.88 (s, 2H), 2.67 (t, 2H, J = 6.0 Hz), 1.63 (p, 2H, J = 4.5 Hz), 1.30-1.24 (m, 18H), 0.88 (t, 3H, J = 6.0 Hz). ¹³C NMR (126 MHz, Chloroform-d) δ 140.6, 130.4, 127.3, 126.7, 122.7, 53.5, 49.8, 47.7, 29.5, 29.4,

29.2, 27.0, 22.5, 13.9. ^{11}B NMR (160 MHz, Chloroform- d) δ 13.65. ESI-MS $[\text{M}+\text{H}]^+$: calcd for $\text{C}_{19}\text{H}_{35}\text{BNO}_2$ 320.2761, found: 320.2318.

2,3-bis(hexadecyloxy)propan-1-amine 3.4

2-(2,3-dihydroxypropyl) isoindoline-1,3-dione (**3.3**) was synthesized as described previously.^[252] This compound (260 mg, 1.17 mmol) was dissolved in 15 mL of anhydrous DMF in a 50 mL round-bottomed flask. Sodium hydride (NaH, 112 mg, 4.68 mmol) was added to this solution under a nitrogen stream. The reaction was allowed to stir at -20°C (acetone ice bath) for 15 min and then at rt for 10 min at which point the solution turned yellow. 1- Bromohexadecane (1.56 g, 4.68 mmol) was added to the solution, which was then stirred at rt overnight. The reaction was then quenched by adding 1 mL of water, and the solvent was removed under reduced pressure. The crude was extracted with dichloromethane from saturated sodium chloride solution (3 x 50 mL). The organic layer was dried with magnesium sulfate, filtered and concentrated to yield crude product as the phthalimide-protected version of **3.4**. Without further purification, the reaction crude was dissolved in 10 mL ethanol along with 2 mL hydrazine monohydrate and heated to reflux overnight at 70°C . The solvent was then removed under reduced pressure. Thin layer chromatography (TLC) using 10% methanol/ dichloromethane as eluant identified the product spot with a R_f value of 0.4 and Ce/Mo and nihydrin stain active. The crude was purified by column chromatography using a gradient solvent system of 2-10%

methanol/dichloromethane to yield amine **3.4** as a pale yellowish oil (221 mg, 35% (over 2 steps)). ^1H NMR (500 MHz, Chloroform-*d*) δ 3.66 – 3.57 (m, 1H), 3.51 – 3.27 (m, 5H), 3.10-3.15 (m, 1H), 2.90 – 2.76 (m, 2H), 1.52 (p, 4H, $J = 4.5\text{Hz}$), 1.36 – 1.17 (m, 52H), 0.88 (t, 6H, $J = 6.0\text{ Hz}$). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 71.9, 71.0, 70.4, 42.7, 31.9, 30.9, 30.1, 29.7, 29.4, 26.1, 22.7, 14.1. DART-MS $[\text{M}+\text{H}]^+$: calcd for $\text{C}_{35}\text{H}_{74}\text{NO}_2$ 540.5719, found: 540.5948.

(2-(((2,3-bis(hexadecyloxy)propyl)amino)methyl)phenyl)boronic acid 3.1

2-Formylphenylboronic acid (55 mg, 0.37 mmol) and triethylamine (51 μL , 0.37 mmol) were dissolved in 5 mL toluene in a 50 mL round-bottomed flask and then amine **3.4** (100 mg, 0.18 mmol) was added. A Dean-Stark trap was fixed to the reaction vessel and filled with 10 mL toluene. The reaction was then allowed to stir for 16 h at 100°C . Next, the reaction mixture was cooled to 0°C in an ice bath and sodium borohydride (28 mg, 0.74 mM) was added slowly, after which the reaction mixture was allowed to stir for 2 h at 0°C . The solvent was next removed through rotary evaporation and the resulting crude was purified with an increasing gradient of methanol/ dichloromethane solvent system from 10% to 100%. Boronic acid lipid **3.1** was obtained as a pale yellow oil (33.9 mg, 28% yield). ^1H NMR (500 MHz, Chloroform-*d*) δ 7.84-7.77 (m, 2H), 7.71-7.64 (m, 2H), 4.06 (s, 2H), 3.90 – 3.81 (m, 2H), 3.80-3.75 (m, 1H), 3.63-3.56 (m, 2H), 3.54-3.46 (m, 2H), 3.44-3.38 (m, 2H), 1.51 (q, 4H, $J = 3.5\text{ Hz}$), 1.29-1.14 (m, 52H), 0.86 (t, 6H, $J = 6.0\text{ Hz}$). ^{13}C NMR (126 MHz,

Chloroform-d) δ 138.6, 130.6, 129.3, 128.5, 127.0, 121.0, 71.2, 65.2, 56.0, 51.3, 31.9, 29.6, 29.3, 26.8, 25.8, 22.7, 14.1, DART-MS $[M+H]^+$: calcd for $C_{35}H_{74}NO_2$ 674.6259, found: 674.3933.

Nile red liposome release studies

Liposome preparation was initiated by weighing out samples of PC, boronic acid lipid **3.1** or **3.5**, and Nile red in separate vials to make 32 mM PC, 5 mM BA lipids and 5 mM Nile red stock solutions in ethanol-free chloroform. Phosphate-buffered saline (PBS, 2 mM, pH 8.0) was chosen as the buffer solution. Heparin lithium salt (10 mg) from porcine intestinal mucosa was added into a 2.08 mL PBS buffer to make a 1,040 U/mL (ie 4.79 mg/mL) stock solution. Benzyl alcohol (0.1%) was added in heparin stock to prevent bacterial growth as suggested by manufacturer. After brief vortexing, the appropriate volumes of the stock solution of each lipid component and Nile red were pipetted into a clean vial per calculations on a 5 mM, 240 μ L total lipid scale, to obtain the desired molar percentage of each component. As an example, for liposomes composed of 10% **3.5** and 85% PC, 5% Nile red, 31.9 μ L PC stock (78.6 μ g), 24 μ L **3.5** stock (3.8 μ g), and 12 μ L Nile red stock (1.91 μ g) were combined. After 30 seconds of vortexing, the chloroform solvent was dried with a nitrogen stream, and the lipids were subsequently dried for at least 3 h under vacuum. Then, the lipids were hydrated with 240 μ L of PBS buffer, incubated on a 60°C water bath for 3 sets of 20 min, with vortexing after each set. Liposomes then underwent freeze-

thaw cycles between a -80°C dry ice-acetone bath and a 60°C water bath for 10 cycles. Vortexing at low speed was performed during each freeze-thaw cycle. Next, liposomes were extruded through a 200 nm membrane filter for 21 passes using a LiposoFast extruder (Avestin, Inc.), and then added to a fresh vial. DLS scans were performed to confirm the formation of stable liposomes. For fluorescence-based release studies, 100 μ L samples of liposome vesicles were added into a sub-micro cuvette cell (Starna Cells, Inc). After an initial fluorescence scan (excitation wavelength of 553 nm, emission wavelength scanned from 560 nm to 580 nm), 0.5 μ L of heparin stock (0.52 U) was added into the cuvette before each fluorescence scan, which was performed 20 times. Data presented in plots indicate emission values at 621 nm. To account for the dilution caused by the addition of heparin solution, blank samples were run by diluting liposomes with the same volumes of water, which were subtracted from the study samples as follows. From the observed fluorescence during the measurement period, a normalized release function that describes the efflux over the period of the experiment was determined according to the equation below to exclude buffer dilution, where $F(tx)$ and $F(bx)$ are the measured fluorescence at titration times x with heparin and buffer accordingly, and $F(t0)$ and $F(b0)$ are initial fluorescence for 2 different set:

$$\% \text{ release} = \frac{F(tx) - F(bx)}{F(t0) - F(b0)} * 100$$

STEM Imaging Studies

Liposome solutions (5 mM) containing either 0% or 10% of boronic acid lipid **3.1**, and the remaining portion consisting of PC (100% or 90%, respectively) were prepared as previously described. One portion of liposomes (100 μ L) was mixed with 10 μ L of a 2,170 U/mL heparin stock as described for the Nile red release studies, and was then incubated at rt for 30 min, while another aliquot was mixed with 10 μ L PBS buffer solution and also incubated at rt for the same amount of time. After that, both portions were diluted to 400 μ M solutions with PBS buffer to reach ideal concentrations for scanning transmission electron microscopy (STEM) studies. A drop (5–10 μ L) from each solution was immobilized onto separate carbon films supported by 200 mesh copper grids. The samples were stained with 5% uranyl acetate for 1 h and then washed three times with distilled water for 10 min and stored in a desiccator overnight prior to imaging. Images were collected using a Zeiss Auriga operating in scanning transmission mode. The electron beam energy was set at 30 KeV, and images were detected using an Everhardt-Thornley SE2 detector.

Sulforhodamine B liposome release studies

Stock solutions were initiated by weighing out samples of PC and boronic acid lipid **3.1** in separate vials to generate separate 32 mM PC and 5 mM **3.1** or **3.5** solutions in ethanol-free chloroform. Phosphate-buffered saline (2 mM, pH 8.0) was chosen as the buffer solution. Heparin lithium salt (10 mg) from porcine intestinal mucosa was added into 1mL

PBS buffer to make 2,170 U/mL stock solution. Benzyl alcohol (0.1%) was added in heparin stock to prevent bacterial growth as suggested by the manufacturer. Sulforhodamine B sodium salt (116.1 mg) was dissolved in 10 mL PBS buffer to make a 20 mM stock solution. After brief vortexing, proper volumes of each lipid stock solution were pipetted into a clean vial per calculations on a 10 mM, 240 μ L total lipid scale to obtain the desired molar percentage of each component. As an example, for liposomes composed of 15% **3.5** and 85% PC, 63.8 μ L PC stock (157.1 μ g) and 72 μ L **3.5** stock (11.5 μ g) were added into a new vial. After 30 seconds of vortexing, the chloroform solvent was dried with a nitrogen stream, and the lipids were subsequently dried for at least 3 h under vacuum. Next, the lipids were hydrated with 240 μ L of the previously described sulforhodamine stock solution and incubated on a water bath at 60°C for 3 sets of 20 min, with vortexing after each set. Liposomes then underwent freeze-thaw cycling between a -80°C dry ice-acetone bath and a 60°C water bath for 10 cycles. Vortexing at low speed was performed during each freeze-thaw cycle. After that, liposomes were extruded through a 200 nm membrane filter for 21 passes using a LiposoFast extruder (Avestin, Inc.) and then added to a fresh vial. Next, liposomes were separated from unencapsulated dye using a Sephadex G-50 size exclusion column (SEC). Liposomes came out in the first or second fraction and were detected by the change in fluorescence upon treatment of an aliquot with triton X-100. DLS scans were performed to confirm the formation of stable liposomes. For fluorescence-based release studies, 100 μ L liposome samples were added into a sub-microcuvette cell (Starna Cells, Inc). After an initial fluorescence scan

(excitation wavelength at 553 nm, emission wavelength scanned from 560 nm to 660 nm), 0.5 μ L heparin stock (1.08 U) was added for each fluorescence measurement before quenching by adding 1 μ L 1% (v/v) Triton-X 100 in PBS to convert liposomes into mixed micelles and trigger rhodamine release. Data presented in plots indicate emission at 606 nm. To account for the dilution caused by the addition of heparin solution, blank samples were run by diluting liposomes with the same volumes of water, which were subtracted from the study samples as follows. From the observed fluorescence during the measurement period, a normalized release function that describes the efflux over the period of the experiment was determined according to the equation below to exclude buffer dilution where $F(t)$, $F(x)$, $F(0)$ are the fluorescence intensity measured after adding Triton X-100, x addition time of heparin stock and initial fluorescence intensity, accordingly. % release = $\left(1 - \frac{F(t) - F(x)}{F(t) - F(0)}\right) * 100\%$

Microplate liposome–heparin binding studies

The wash buffer used in this study was 10 mM PBS at pH 8.0. 10 μ g Heparin–biotin sodium salt purchased for Sigma-Aldrich (~ 15000 kDa) was added into 10 mL PBS buffer to prepare a 1 μ g/mL stock solution. Liposome solutions (2 mM) consisting of 1% rhodamine L- α -phosphatidylethanolamine (Rd-PE), either 0% or 20% of boronic acid lipid **3.1**, and the remaining portion consisting of PC (99% or 79%, respectively) were prepared as previously described. Next, opaque streptavidin-coated 96-well microplate wells were

washed with 250 μ L buffer for 30 min. After removal of buffer, 100 μ L of heparin–biotin solution was added into study wells. Additional negative controls were run in which wells were treated with the different liposome compositions, but not with heparin–biotin, and experiments were otherwise run the same. After 1 h of incubation, these solutions were removed, and the wells were washed three times with 250 μ L wash buffer for 5 min. Next, aliquots of liposomes were diluted into wash buffer to a total volume of 100 μ L, producing solutions of 0, 20, 40, 80, 100, 200, 400, 500, 600, 800, and 1000 μ M. These solutions were added to separate wells of the microplate functionalized with heparin. After 2h of incubation, these solutions were removed, and the wells were washed with 250 μ L wash buffer three times for 5 min each. Finally, 100 μ L of wash buffer was added to each well, and the microplate was analyzed using a Biotek Synergy 2 microplate reader, and the fluorescence intensity was repeatedly measured at excitation wavelength of 530 $\pm$ 20 nm and emission wavelength of 590 $\pm$ 20 nm.

Cell culture and fluorescence microscopy

Melanoma A375 cells, obtained from ATCC[®] (Manassas, VA), were cultured in a humidified incubator under 5% CO₂ in DMEM medium supplemented with 10% of Fetal Bovine Serum, 50 U/mL penicillin, and 50 μ g/ml streptomycin (Invitrogen). Two days prior to the experiment, A375 cells were plated at a seeding density of 1×10^4 cells per well on a glass coverslip. Liposomes containing 0.08% rhodamine L- α -phosphatidylethanolamine (Rd-PE)

in the absence or presence of 10% of boronic acid lipid **3.1**, with the rest of the liposome consisting of PC were incubated with cells at a concentration of 1 mM for 1 h at 37°C. Liposome preparation was conducted under similar conditions as described above, except 10 mM PBS pH 7.4 was used as the buffer. After incubation, cells were washed four times with PBS containing 1 mM MgCl₂ and 100 mM CaCl₂ (PBS⁺⁺), fixed for 30 min in 4% paraformaldehyde and mounted with ProLong Diamond Antifade Mountant with DAPI (Invitrogen). Images were acquired on a confocal laser scanning microscope (Zeiss LSM 710) with 63x objectives using Zen2 blue edition software. Contrast and brightness settings were chosen so that all pixels were in the linear range. Images are the product of fourfold line averaging.

4. Bisboronic acid liposomes for selective binding of sialic acids and enhanced cancerous cell delivery

4.1 Introduction

4.1.1 Overview

In the previous chapter, lipids with a single boronic acid headgroup were synthesized and studied for carbohydrate triggered release and enhanced cellular delivery. In this chapter, more sophisticated lipids containing two boronic acids were developed with the goal of selective binding interactions with sialic acid-containing carbohydrates that are often overexpressed on cancer cell surfaces. Here, we were inspired by previous work in the field showing that rigid bisboronic acid compounds can yield enhanced selectivity for particular carbohydrate targets. To translate this work for liposome delivery, we designed and synthesized bisboronic acid lipids containing with a small library of selected aromatic spacers to control the distance between the two boronic acid units.[62] To study the carbohydrate-binding properties of bisboronic acid liposomes, we first developed a three-component fluorescent competition assay using Alizarin Red S to evaluate the binding affinity toward *N*-acetylneuraminic acid. Then, liposomes containing bisboronic acid lipids were investigated in hydrophobic and hydrophilic fluorescent dye triggered release study using the sialic acid-rich glycoprotein mucin. Next, the most effective bisboronic acid lipid from these prior studies was chosen for liposomal cell delivery experiments using fluorescence microscopy. In these studies,

dose-dependent cell labelling was observed in melanoma cell lines based on the percentage of bisboronic acid lipid within liposomes, which indicates the potential of synthetic bisboronic acid lipids for selective drug delivery.

4.1.2 Structure, property and biological significance of sialic acid

As depicted in **Figure 4.1**, sialic acids compose a family of around 40 derivatives of the nine-carbon sugar neuraminic acid, which is the unsubstituted form of this sugar that does not exist in nature.[253-255] The most abundant sialic acid is *N*-acetylneuraminic acid (Neu5Ac) where the amino group is acetylated. Due to the acidity of the neutral form of this molecule ($pK_a = 2.6$), the carboxyl group at position 1 is negatively charged under physiological conditions.

Due to their size, hydrophilicity, acidity and its negative charge, sialic acids play important roles in cellular and molecular recognition events.[256-258] These sugars can bind and transport positively charged molecules (such as calcium ions (Ca^{2+})), and also stabilize the conformations of enzymes or cell membranes proteins.[259] Perhaps the most interesting feature of sialic acids is their presence in many oncofetal carbohydrate antigens. It is well known that tumor cell surfaces are decorated with abnormal glycosylation makeup in the form of overexpressed naturally occurring or pathophysiological oligosaccharides.[260] It is estimated that there are as many as 10^9 sialic acid residues on multiple sialylated antigens per tumor cell.[261] The alteration, in both structure and abundance, of sialic acids has been correlated with malignant and

metastatic phenotypes in epithelial-derived cancers, such as gastric, colon, pancreatic, liver, lung, prostate, and breast cancers.[262] The high level of sialylation has an immunosuppressive effect since it prevents cells from degradation by masking subterminal galactose residues.[263] As a result, strategies that can target cells based on their sialic acid expression may be beneficial for selective tumor diagnosis as well as treatment.

Due to the overexpression of sialic acids on diseased cells, significant effort has gone into exploiting this phenomenon for the selective labeling of diseased cells. For example, the Bertozzi lab reported a highly selective reaction of ketones with aminooxy groups that can differentiate subtle modifications in sialoside metabolism and ultimately enable selective delivery of magnetic resonance contrast reagents to tumor cells.[264] The Peters lab designed and synthesized two novel lanthanide ion ligands consisting of diethylenetriamine pentaacetic acid (DTPA)-bisamide that are capable of molecular recognition of sialic acid residues by boronic functional moiety.[265] Djanashvili and coworkers have demonstrated *in vivo* targeting based on the recognition of overexpressed sialic acid by a phenylboronic acid-based imaging reporter in aid of low molecular weight Gd complex.[266] As a major topic of this dissertation, a more in-depth discussion of selective boronic acid materials for sialic acids is included in the section below.

4.1.3 Selective detection of carbohydrates using bisboronic acid materials

Initially demonstrated in the seminal work done by Yoon and Czarnik in 1992, boronic acid-based materials can act as fluorescent sensors of carbohydrates.[47] In the two decades since, the development of boron-based materials for monosaccharides, polysaccharides and cell surface glycans has been a focus in carbohydrate research and material science. The Shinkai group reported a landmark study describing a selective glucose sensor **4.1** (**Figure 4.2**) with chiral binaphthol core and aminoboronate that uses a photoinduced electron transfer (PET) signal transduction scheme.[267] This system can differentiate between enantiomers of glucose and overcome the low affinity of single boronic acid sensors for glucose at physiological pH by using a bisboronic acid. The Singaram lab has developed a dye/quencher system using the anionic dye 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (pyranine) and bisboronic acid bipyridinium salt **4.2** for the selective binding of glucose.[268]

Besides the ability to recognize monosaccharides, boronic acid materials have been explored for molecular interactions with cellular surface glycan in physiological settings. Boronic acids can form boronate esters reversibly with polyol motifs on sugars and selectivity towards certain carbohydrate can arise from the intrinsic orientation of hydroxyl groups on the rigid carbocyclic skeleton. The biggest drawback of using boronic acids is that they exhibit a prominent preference for binding furanosides, while possessing low binding affinity towards hexopyranosides, which are the major components of cell surface glycoconjugates.[269] To achieve selectivity towards pyranose sugars, the Hall lab

reported a rationally designed library of synthetic receptors of type **4.3** containing a peptide backbone that can act as both a hydrogen bonding donor or acceptor with two benzoboroxole groups attached on diaminopropionic acid residues in the central amino acid position.[59] The function and geometrical diversity of these structures are achieved by twenty different natural and synthetic amino acids serving as spacers in the middle of the peptide backbone and the acyl capping group consisting of a selection of twenty carboxylic acids. Selectivity in the binding of the TF antigen disaccharide was discovered upon screening a library of 400 peptidyl bis(boroxole) receptors using a competitive enzyme-linked immunosorbent assay (ELISA) assay.

The Wang lab additionally developed a family of anthracene-based fluorescent bisboronic acid sensors of type **4.4**, for which a change in fluorescence intensity is caused by boronic ester formation upon the binding of carbohydrates along with the masking of the nitrogen lone pair of electrons.[62] Their diboronic acid compounds with the proper spatial arrangement of the two boronic acid headgroups were pruned to multiple pairs of diols and showed highly specific affinity for the target carbohydrate Sialyl Lewis X.

Other boronic acid-based compounds that are not bisboronic acids have also shown selectively towards certain carbohydrate. For example, the Miyahara lab developed a group of heterocyclic boronic acids possessing high affinity and selectivity for sialic acids.[270] Among them, 5-boronopicolinic acid and 3-propionamidophenylboronic acid have proven to be strong sialic acid-binders under acidic pH conditions of hypoxic tumoral microenvironment.

4.2 Design and synthesis of bisboronic acid lipids

In this project, we set out to develop bisboronic acid lipids that could translate selective carbohydrate binding properties to a membrane environment as a means for achieving the selective delivery of liposomes to sialic acid-presenting diseased cells. Toward this end, the design and synthesis of bisboronic acid lipids is shown in **Figure 4.3**. First, a Wulff-type type *ortho*-(alkylaminomethyl)phenylboronic acid unit was introduced on the lipid scaffold. This was done through a reductive amination reaction between dodecylamine **4.5** and (2-formylphenyl)boronic acid with the aid of a Dean-Stark trap to remove water that is produced. Since the phenyl ring is known to stabilize the boronic acid through hyperconjugation effects and the ammonium moiety (in the protonated form of **3.5**) serves as an electron withdrawing group, our synthetic boronic acids are expected to possess increased Lewis acidity with lower pK_a value while also exhibiting higher affinities towards carbohydrates at physiological pH.[12]

As mentioned in the previous section, cancerous cell surfaces have aberrant glycosylation patterns and overexpression of sialic acids is one of the signature features of tumor cells. To achieve high selectivity for specific carbohydrates, our strategy was to vary the identity and length of the linker connecting the two boronic acids backbone to probe for selectivity towards sialic acids. For this purpose, compound **3.5** was treated with aromatic dibromide compounds in the presence of potassium carbonate to give bisboronic acid lipids **4.6a-e**. As listed in **Table 4.1**, aromatic spacers with different length,

rigidity and geometry that were used to link the two boronic acid lipids include *o*-xylene, *m*-xylene, *p*-xylene, 2,6-dimethylnaphthalene and 4,4'-dimethyl-1,1'-biphenyl.

4.3 Competition assay of dye alizarin red s to analyze sialic acid binding

Following the synthesis of bisboronic acid lipids **4.6a-e**, we developed a competitive binding assay of boronic acids with the aid of fluorescent dye Alizarin Red S (ARS) to evaluate their affinity towards sialic acid. *N*-Acetylneuraminic acid was chosen as a representative carbohydrate model since it is the most predominant of the family of sialic acids and can be found attached to many mammalian cells, human cells included.[271] ARS has been used as a reagent to measure the concentration of boric acid, since the active protons of the hydroxyl groups among hydroxyanthraquinone compounds can form intra/intermolecular hydrogen bonds with ketones and result in significant fluorescence quenching, with fluorescence restored when the catechol diol of ARS binds to a boronic acid.[272-276]

The fluorescence intensity changes in this three-component system can be used to measure the binding affinity of bisboronic acid lipids qualitatively and quantitatively. As shown in **Figure 4.4**, two competing equilibria are present in the fluorescence assay system. The first equilibrium is between the boronic acid and the fluorescent indicator ARS, which can be directly measured by an increase in fluorescence caused by boronate formation. When Neu5Ac is added to the system, the second equilibrium involves the binding of the bisboronic acid lipid to the added carbohydrate, which occurs through

displacement of the previously bound ARS and leads to a decrease in fluorescence intensity through ARS release and protonation.

A representative full titration curve for treatment of *m*-xylene bisboronic acid lipid **4.6b** with ARS and then Neu5Ac is shown in **Figure 4.5**. To overcome the solvent incompatibility of hydrophobic bisboronic acid lipid **4.6b** and hydrophilic guests ARS and Neu5Ac, bisboronic acid lipids were incorporated into PC-based liposomes in PBS buffered aqueous environment. Liposomes were prepared using conventional thin layer film method using a standard protocol including hydration, free-thaw and size extrusion. First, nine portions of 0.5 μL of a 2 mM ARS solution were added to 100 μL of a 5mM solution of PC-based liposomes containing 0 or 10% bisboronic acid lipids **4.6b** and fluorescence intensity was measured after adding each aliquot of ARS. Following this, Neu5Ac was added to competitively bind to bisboronic acid liposomes by displacing ARS. In this phase of the experiment, nineteen 0.5 μL aliquots of a 50mg/mL Neu5Ac solution were added to the system, which led to a drastic decrease in fluorescence intensity. At the endpoint, the total amount of liposomes with *m*-xylene bisboronic acid lipid **4.6b**, ARS, and Neu5Ac were 5×10^{-7} mol, 5×10^{-8} mol, 9×10^{-9} mol, and 3.2×10^{-9} mol.

in the results shown in **Figure 4.5**, fluorescence intensity was found to steadily increase with the addition of ARS. Then, with the introduction of Neu5Ac, some bound ARS was replaced by carbohydrate and fluorescence was quenched to almost 50% of the maximum, after which it plateaued. These results indicate the efficacy of synthetic bisboronic acid lipid **4.6b** for binding both ARS and Neu5Ac. Also, the competitive three-

component fluorescence binding assay was proven to be effective for measurement of binding interactions.

This competitive three-component fluorescence assay was applied to examine the binding affinity of all bisboronic acid lipids **4.6a-e** toward Neu5Ac. Instead of titrating ARS solution and measuring the fluorescence intensity upon each addition, 10 μ L of 2mM ARS solution was added at once to 5mM solutions of PC liposomes containing 10% bisboronic acid lipids **4.6a-e** to reach maximum fluorescence intensity. Then, 1 μ L of Neu5Ac was titrated into the system as previously described, with the results shown in **Figure 4.6**. Here, all of the bisboronic acid liposomes exhibited a decrease in fluorescence intensity when Neu5Ac was introduced and plateaued before the endpoint. Among them, *o*-xylene bisboronic acid lipid **4.6a** yielded the most significant fluorescence drop (~70%), followed closely by *m*-xylene bisboronic acid lipid **4.6b**, while 2,6-naphthyl bisboronic acid lipid **4.6d** exhibit the lowest fluorescence decrease (~20%). In all of these data, we have deducted the change in fluorescence caused by dilution, which is measured by titration with buffer instead of Neu5Ac. Another set of negative control involved the use of 100% PC liposomes, for which data were not plotted since no fluorescence increase occurred after the addition of ARS, confirming that the bisboronic acids were responsible for the binding of ARS. These experiments indicated that bisboronic acid lipids with different spacers possess variable affinities towards Neu5Ac and that the most effective were *o*-xylene bisboronic acid lipid **4.6a** and *m*-xylene bisboronic acid lipid **4.6b**.

4.4 Fluorescence-based dye leakage assay

4.4.1 Porcine mucin triggered bisboronic acid liposomes release of sulforhodamine b

Following the sialic acid binding study, we next moved to test triggered release properties using bisboronic acid lipids **4.6a-e** incorporated into phospholipid vesicles through titration with porcine mucin, shown in **Figure 4.7** (hydrophilic dye) and **Figure 4.10** (hydrophobic dye). As discussed in **section 2.1.2** mucin is a glycoprotein that is primarily composed of carbohydrate units (60 - 80% of the molecular weight).[164, 165] The target carbohydrate of this project, sialic acid, is the terminal glycan on *O*-glycosylated sugar branches and makes up around 1.2% of the total composition of mucin.

We first studied the capabilities of bisboronic acid liposomes for releasing hydrophilic compounds. To do so, liposomes containing 10% bisboronic acids **4.6a-e** were loaded with the fluorescent dye Sulforodamine B. **Figure 4.8** shows the efflux curve for a typical trigger release measurement from PC-based vesicles, where background fluorescence was measured without the addition of mucin. Subsequent measurements were taken at 80 μ g intervals of mucin addition for 20 additions. At the end of the titration, 1 μ L of 1%(v/v) Triton X-100 (TX100) was added. As one of the most widely used nonionic surfactants for permeabilization of membrane bilayers, TX100 leads to 100% leakage of the vesicle contents. Specifically, liposomes containing *m*-xylene bisboronic acid lipid **4.6b** spacer showed the greatest amount of release of Sulforhodamine B (~ 50% signal increase). On the other hand, liposomes containing 2,6-naphthyl bisboronic acid

lipid **4.6d** showed the least amount of release of Sulforhodamine B (less than 5% signal increase). Background release (~10% signal increase) of liposomes containing 0% bisboronic acid lipids was observed. The reason why more dye leakage was found in liposomes containing only PC than liposomes containing 2,6-naphthyl or 4,4-biphenyl bisboronic acid **4.6d/e** was unclear.

Results obtained for DLS measurements indicated significant liposome disruption upon mucin treatment. As illustrated in **Figure 4.9**, liposomes containing either 0% or 10% of any of the bisboronic acid lipids showed uniform liposome formation with Z average values of approximately 200 nm before titration with mucin, supporting the formation of stable liposomes of the expected sizes using these compounds. After addition of mucin, PC liposomes lacking any bisboronic acid lipids showed no size change, while nearly all the liposomes containing 10% bisboronic acid lipids yielded a major size increase. In line with previous results, *m*-xylene bisboronic acid lipid **4.6b** yielded the most significant size change (~1500nm size increase), which matched the data from fluorescence efflux studies. Increases in particle sizes detected by DLS are often an indication of morphology changes of self-assembled lipids.

There are many possible explanations for this phenomenon. First, boronic acid lipids located on different liposomes can be brought into close proximity when interacting with mucin, which, along with the destabilization of membrane bilayers, can lead to liposomal fusion and aggregation. Second, conformational changes that result from the formation of boronate esters as a result of binding between bisboronic acid lipids and diol

units on mucin could also drive changes in lipid self-assembly. The sp^3 hybridized C-N bond located within the bisboronic acid lipid structure can also rotate to enhance the interaction with mucin. This can cause turbulence of the sample caused by destabilization of the liposome bilayer structure.

4.4.1 Porcine mucin-triggered release of Nile red from bisboronic acid liposomes

To mimic non-polar drugs, the hydrophobic fluorophore Nile Red was also studied. As can be seen in **Figure 4.8**, titration with mucin also led to varied levels of fluorescence decreases depending upon the particular bisboronic acid lipid that was incorporated into liposomes at 10% composition. Specifically, liposomes containing bisboronic acid with the *m*-xylene spacer (**4.6b**) showed maximum release of Nile red (~ 25% signal decrease). On the other hand, liposomes containing bisboronic acid lipid with the 4,4-biphenyl spacer (**4.6e**) showed minimal release of Nile red (~12% signal decrease). Overall, the results matched up well with the binding abilities observed in the sialic acid dye displacement assays and the Sulforhodamine B release experiments. Minimal background release (~5%) from liposomes containing 0% bisboronic acid lipids was observed. These data provide evidence that the bisboronic acid units presented on the liposomes can trigger Nile red to release to a significantly different extent, indicating their varied binding affinity towards the glycoprotein mucin.

We have also observed that the extent of release from liposomes can vary depending on the concentration of liposomes used for studies. Thus, we also performed

experiments in which the total concentrations of liposomes were decreased to 500 μ M (**Figure 4.9**). In these experiments, while the trends for release were similar among the different bisboronic acids, the overall extent of release was found to be greater for all samples, including controls. Liposomes containing bisboronic acid lipid **4.6b** with a *m*-xylene spacer showed maximum release of Nile red (~ 50% signal decrease). On the other hand, liposomes containing bisboronic acid lipid **4.6e** with the 4,4-biphenyl spacer showed minimal release of Nile red (~27% signal decrease). Compared to the previous study of 5mM liposomes, background release was more significant in this case since an ~20% decrease in fluorescence intensity was observed using PC liposomes lacking bisboronic acid lipids.

The reason why more significant release was observed in all samples, including controls, is perhaps more complex than can be succinctly explained in this dissertation. This could be explained by the fundamental increase of micellar size and the polydispersity upon buffer dilution of phospholipids bilayer solutions.[277] Furthermore, the author believes this may have something to do with changes in osmotic pressure when the liposomes were diluted to 500 μ M. The morphology of lipid bilayers can vary in response to environmental changes and osmotic pressure is one of the most-studied factors involved in bilayer homeostasis.[278, 279] The osmotic pressure on each individual liposomal vesicle is different when the same amount of mucin glycoprotein is added to a 5mM liposome stock as opposed to a 500 μ M liposome stock. Subjecting dilute liposomes to high osmotic pressure could disrupt the integrity of their spherical bilayer

structures and result in more content release.

Results from DLS experiments also indicated changes in liposome particle sizes upon mucin treatment, as shown in **Figure 4.10**. The average size of mucin is around 800nm. In these studies, all liposomes consisting of a particular bisboronic acid lipid of type **4.6a-e** ranging from 0-10% mixed with PC yielded the expected sizes following extrusion through a 200nm filter during liposome preparation. This indicates stable liposomes were correctly formed when bisboronic acid lipids were mixed with PC at these percentages. Upon treatment with mucin, liposomes containing 0% bisboronic acid lipids exhibited minimal change in size and polydispersity index (the latter data not shown), which matches the lack of change in Nile red fluorescence from release studies. Solutions of 5mM liposomes containing 10% bisboronic acid lipids showed slight changes (~10 to 60nm) in sizes upon mucin addition, while 500 μ M samples of bisboronic acid liposomes had more significant increase in sizes (~100 to 150nm) and a profound jump in polydispersity index (~0.6). However, size changes were not as profound as the studies of liposomes containing monoboronic acid lipid **3.5** triggered by heparin titration in chapter two.

Several factors may contribute to this phenomenon. First, since there are two boronic acid headgroups presented on one bisboronic acid lipid and they are linked with a rigid aromatic space in a fixed geometry, molecular orientation is limited when lipids interact with polyols in carbohydrates. Second, bisboronic acid lipids generally yielded diminished fluorescence intensity decreases compared to mono-boronic acid lipids, which

suggests that less reorganization of lipids is occurring when binding to mucin. Despite the diminished changes observed during DLS results, the efflux curves obtained from fluorescence titration provided evidence of carbohydrate binding when sufficient boronic acids are presented on the liposome surface that leads to vesicle leakage and dye release. A more precise and intuitive way to understand the changes of liposome size and structure upon titration with mucin is STEM, for which experiments will be done in the future.

4.5 Cellular delivery studies

With successful results from triggered-release studies, we next set out to assess cellular labeling and delivery of bisboronic acid liposomes to melanoma A375 cells, as depicted in **Figure 4.14**. These experiments were done in collaboration with the Barrera lab in the Department of Biochemistry and Cellular and Molecular Biology at UTK. In these experiments, we incubated liposomes containing bisboronic acid lipids **4.6b** with cells to allow the interaction between cell surface glycans and boronic acid moieties. The close proximity resulting from this interaction along with the self-assembled characteristics of the liposomes, including fluorescent Rd-PE, is expected to enhance entry into cells leading to increased fluorescence in microscopy experiments. Since bisboronic acid lipids containing the *m*-xylene spacer (**4.6b**) showed relatively high binding affinity to sialic acids and the most significant liposomal dye release, this compound was selected for cell delivery studies. Cells were incubated with 1mM of LUVs containing 0.08% Rd-PE, in the

presence of liposomes containing 0%, 2%, 5% 10% of **4.6b**, and otherwise composed of PC, for 2 hours at 37°C. After proper washing, cells were fixed and mounted on glass slides for confocal laser scanning microscope.

Representative images of at least 3 experiments are shown in **Figure 4.15**. In these images, the orange signal arises from the Rd-PE channel, while the blue shows DAPI staining of nuclei to show the presence of live cells. Cells treated with liposomes containing **4.6b** (**Figure 4.15B-D**) resulted in significant fluorescence compared to those of control studies lacking **4.6b** (**Figure 4.15A**). More specifically, a dose-dependent response was observed in which liposomes containing 10% of **4.6b** had the most intense fluorescence signal while liposomes containing 2% of **4.6b** had the least fluorescence signal. Regarding cytotoxicity, neither liposomes containing 2%, 5% or 10% of **4.6b** showed any cytotoxicity or caused any cell morphology changes, as judged from the images. These results provide strong evidence that our synthetic bisboronic acid lipid **4.6b** can enhance cellular delivery of liposomes at a low dose while avoiding cell damage.

4.6 Summary and future outlook

In conclusion, we have designed, synthesized and analyzed novel bisboronic acid lipids **4.6a-e**, which showed variable binding abilities towards sialic acids, and were effective for triggering release of both hydrophobic and hydrophilic compounds and enhancing delivery to cancer cells. Despite the unnatural structures of synthetic

bisboronic acid lipids, we validated their ability to form stable and uniformly sized liposomes through DLS upon incorporation into PC liposomes. We observed wide-ranging extents of content release through changes in fluorescence intensity when liposomes included bisboronic acid lipids **4.6a-e**, which supports their varied binding affinities towards sialic acids. We then selected compound **4.6b** and studied its potential as a drug delivery agent to cancer cells, since **4.6b** showed the strongest interactions with Neu5Ac and sialic acids-rich glycoprotein mucin. To do so, melanoma cells were treated with fluorescent liposomes containing **4.6b** and strong labeling was observed through confocal microscopy in a short period of time with minimal cytotoxicity.

There are additional experiments that can be done to further explore the potential of bisboronic acid lipids **4.6a-e**. We have investigated the binding affinity of bisboronic acid liposomes towards Neu5Ac, and shown that structural alterations lead to variable binding properties. To build on this work, we can screen for the selectivity of bisboronic acid liposomes towards different carbohydrates, and particularly those that are additionally present in mucin-1 such as glucose, galactose, and fucose. It would be interesting to see if the compounds that yielded the highest binding affinity towards Neu5Ac would also exhibit enhanced selectivity for this sugar. Apart from the selectivity arising from the spatial arrangement between the two boronic acid units within the bisboronic acid, potential selectivity towards sialic acid could also benefit from the intrinsic favored diol location on Neu5Ac. The seminal work reported by the Wang lab has

shown that the K_{eq} of the phenylboronic acid sialic acid interaction is five times higher than that with glucose.[5]

More sophisticated cellular delivery studies can also be done to obtain a greater understanding of how bisboronic acid liposomes behave in biological environments. To evaluate the impact of sialic acids located on the cell surface, we could remove them first and compare the difference in cell entry using bisboronic acid liposomes. One way to do that is to use commercially available sialidase enzymes, which can cleave terminal sialic acid residues that are linked to monosaccharides, oligosaccharides, glycolipids, or glycoproteins via glycosidic bonds. Several research groups have reported that cell entry was minimal using boronic acid material after cells were treated with sialidase.[64, 270, 280-282]

In conclusion, we have designed bisboronic acid lipids that showing varied binding affinity towards sialic acids based on the specific spatial arrangement of the boronic acid moieties. Liposomes containing these compounds have also been shown to be effective for triggered release of encapsulated contents in the presence of mucin-1 as well as enhanced cell delivery. Further work is planned to probe the carbohydrate-binding selectivity of these structures. This work presents a promising platform for selective cancer cell drug delivery.

4.7 Materials and method

General experimental. Reagents and solvents were generally purchased from Acros, Aldrich or Fisher Scientific and used as received. Mucin from porcine stomach was purchased from Sigma-Aldrich, Inc. L- α -Phosphatidylcholine (mixed isomers from egg) and L- α -phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) were purchased from Avanti Polar Lipids, Inc. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using Varian Mercury 300 or 500 MHz spectrometers. Mass spectra were obtained with JEOL DART-AccuTOF or Applied Biosystems/ QStar Elite HPLC—QTOF spectrometers. Fluorescence titration experiments were performed using a Cary Eclipse Fluorescence Spectrophotometer from Agilent Technologies. Water for liposome preparation and triggered -release studies was purified using a Milli-Q filtration system. Data were plotted and curve-fitted using SigmaPlot 13. Dynamic Light scattering (DLS) measurements were performed to study the size and stability of liposomes using a Malvern Zetasizer Nano ZS instrument equipped with a He-Ne 633 nm laser and a temperature controller at a scattering angle of 173°. All plots represent the average of at least 3 experiments with error bars denoting +/- the standard error for each set of measurements.

Synthesis

Bisboronic acids with aromatic spacers 4.6a-e

Compound **3.5** (2-((dodecylamino)methyl)phenyl)boronic acid was prepared following the protocol provided by Best and coworkers, and in chapter 3 of this dissertation.[71] Compound **3.5** (100mg, 0.3 mmol) was dissolved in 10 mL dry dimethylformamide (DMF), together with the appropriate dibromide corresponding to each spacer (0.16 mM, 0.53 equiv), potassium carbonate (65mg, 0.47mmol) and potassium iodide (2 mg). The reaction mixture was heated to reflux at 90°C for 12 hr. The insoluble materials were then filtered off, and the filtrate was washed with 3 x 100 mL water, and then with 100 mL 1M HCl, 100 mL saturated NaHCO₃ and 100 mL brine. The solvent was evaporated in vacuo and the residue was purified on a silica gel column, eluting with hexane/ EtOAc 9:1 to give the products. The retention factor (R_f) was ~ 0.8 on TLC plate using 25% hexane/ EtOAc eluant mixture.

(((1,2-phenylenebis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene))diboronic acid 4.6a

Product was obtained as white powdery solid (106mg, 0.144mmol, 96%yield). ¹H NMR (500 MHz, CDCl₃) δ 7.39-7.36 (m, 3H), 7.33 – 7.28 (m, 6H), 7.25-7.20 (m, 2H), 3.55 (s, 4H), 3.54 (s, 4H), 2.40 (t, *J* = 7.3 Hz, 4H), 1.58 – 1.45 (m, 4H), 1.33-1.24 (m, 36H), 0.90 (t, *J* = 7.3 Hz, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 140.16, 138.38, 128.75, 128.52, 128.05,

126.61, 58.27, 58.04, 53.41, 31.94, 29.69, 29.66, 29.53, 29.37, 27.28, 27.00, 22.71, 14.14.

(((1,3-phenylenebis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene))diboronic acid 4.6b

Product was obtained as white powdery solid (108mg, 0.147mmol, 98%yield). ^1H NMR (500 MHz, CDCl_3) δ 7.43-7.37 (m, 4H), 7.34-7.29 (m, 4H), 7.27-7.22 (m, 4H), 3.58 (s, 8H), 2.44 (t, $J = 7.3$ Hz, 4H), 1.58 – 1.45 (m, 4H), 1.37-1.17 (m, 36H), 0.93 (t, $J = 7.3$ Hz, 6H). ^{13}C NMR (126 MHz, CDCl_3) δ 140.18, 139.81, 129.27, 128.76, 128.08, 127.84, 127.23, 126.63, 77.28, 77.03, 76.78, 58.33, 53.45, 31.97, 29.73, 29.71, 29.59, 29.41, 27.34, 27.08, 22.74, 14.16.

(((1,4-phenylenebis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene))diboronic acid 4.6c

Product was obtained as white powdery solid (105mg, 0.142mmol, 95%yield). ^1H NMR (500 MHz, CDCl_3) δ 7.39-7.25 (m, 4H), 7.32-7.28 (m, 6H), 7.25-7.20 (m, 2H), 3.55 (s, 4H), 3.54 (s, 4H), 2.40 (t, $J = 7.2$ Hz, 4H), 1.51 (p, $J = 6.8$ Hz, 4H), 1.30-1.23 (m, 36H), 0.90 (s, 6H). ^{13}C NMR (126 MHz, CDCl_3) δ 140.16, 138.38, 128.75, 128.52, 128.05, 126.60, 77.26, 77.00, 76.75, 58.27, 58.04, 53.41, 31.94, 29.69, 29.66, 29.53, 29.37, 27.28, 27.00, 22.71, 14.14.

(((naphthalene-2,6-diylbis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene))diboronic acid 4.6d

Product was obtained as yellow oil (39mg, 0.05mmol, 33%yield). ^1H NMR (500 MHz, Chloroform-*d*) δ 7.68-7.64 (M, 4H), 7.45-7.42 (m, 2H), 7.31-7.29 (m, 4H), 7.25-7.20 (m, 2H), 7.16-7.11 (m, 2H), 3.60 (s, 4H), 3.51 (s, 4H), 2.36 (s, 4H), 1.49-1.42 (m, 4H), 1.20-1.12 (m, 36H), 0.80 (t, J = 7.2 Hz, 6H). ^{13}C NMR (126 MHz, cdcl_3) δ 140.05, 137.21, 132.58, 128.79, 128.10, 127.48, 127.25, 126.97, 126.67, 77.27, 77.01, 76.76, 58.48, 58.28, 53.45, 31.94, 29.68, 29.66, 29.64, 29.54, 29.37, 27.30, 27.00, 22.71.

(((1,1'-biphenyl)-4,4'-diylbis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene))diboronic acid 4.6e

Product was obtained as yellow oil (68mg, 0.084mmol, 56%yield). ^1H NMR (500 MHz, Chloroform-*d*) δ 7.57-7.51 (m, 4H), 7.46-7.39 (m, 6H), 7.34-7.31 (m, 4H), 7.25-7.23 (m, 2H), 3.60 (d, J = 3.1 Hz, 8H), 2.45 (t, J = 7.1 Hz, 4H), 1.55 (p, J = 7.6, 7.1 Hz, 5H), 1.31-1.25 (m, 36H), 0.90 (t, J = 6.7 Hz, 6H). ^{13}C NMR (126 MHz, cdcl_3) δ 140.08, 139.50, 138.99, 129.24, 129.11, 128.76, 128.11, 127.42, 126.69, 77.27, 77.02, 76.76, 58.31, 57.96, 53.47, 31.95, 29.70, 29.67, 29.55, 29.37, 27.29, 27.03, 22.71, 14.15.

Competition binding assay of bisboronic acid liposomes through displacement of ARS with Neu5Ac

Liposome preparation was initiated by weighing out samples of PC, bisboronic acid lipid **4.6a-e** or compound **3.5**, and Nile red in separate vials to make 32 mM PC, 5 mM BA lipids stock solutions in ethanol-free chloroform. Phosphate-buffered saline (PBS, 10 mM, pH 8.0) was chosen as the buffer solution. Solutions of 2mM ARS and 50 mg/L Neu5Ac were also prepared in PBS buffer. After brief vortexing, the appropriate volumes of the stock solution of each lipid component and Nile red were pipetted into a clean vial per calculations on a 5 mM, 240 μ L total lipid scale, to obtain the desired molar percentage of each component. As an example, for liposomes composed of 10% **4.6a** and 90% PC, 33.8 μ L PC stock (83.2 μ g), 24 μ L **4.6a** stock (3.8 μ g) were combined. After 30 seconds of vortexing, the chloroform solvent was dried with a nitrogen stream, and the lipids were subsequently dried for at least 3 h under vacuum. Then, the lipids were hydrated with 240 μ L of PBS buffer, incubated on a 60°C water bath for 3 sets of 20 min, with vortexing after each set. Liposomes then underwent freeze-thaw cycles between a -80°C dry ice-acetone bath and a 60°C water bath for 10 cycles. Vortexing at low speed was performed during each freeze-thaw cycle. Next, liposomes were extruded through a 200 nm membrane filter for 21 passes using a LiposoFast extruder (Avestin, Inc.), and then added to a fresh vial. DLS scans were performed to confirm the formation of stable liposomes. For fluorescence-based release studies, 100 μ L samples of liposome vesicles and 10 μ L ARS solution were added into a sub-micro cuvette cell (Starna Cells, Inc). After an initial

fluorescence scan (excitation wavelength of 425 nm, emission wavelength scanned from 500 nm to 700 nm), 1 μ L of Neu5Ac solution was added in the cuvette before each fluorescence scan, which was performed 19 times. Data presented in plots indicate emission values at 593 nm. To account for the dilution caused by the addition of Neu5Ac solution, blank samples were run by diluting liposomes with the same volumes of buffer solution, which were subtracted from the study samples as follows. From the observed fluorescence during the measurement period, a normalized release function that describes the efflux over the period of the experiment was determined according to the equation below to exclude buffer dilution, where $F(tx)$ and $F(bx)$ are the measured fluorescence at titration times x with Neu5Ac and buffer accordingly, and $F(t0)$ and $F(b0)$ are initial fluorescence for the 2 different sets:

$$\% \text{ release} = \frac{F(tx) - F(bx)}{F(t0) - F(b0)} * 100$$

Nile red Release studies upon titration of bisboronic acid liposomes with mucin

Liposome preparation was initiated by weighing out samples of PC, a specific boronic acid lipid of type **4.6a-e** or **3.5**, and Nile red in separate vials to make 32 mM PC, 5 mM BA lipids and 5 mM Nile red stock solutions in ethanol-free chloroform. Phosphate-buffered saline (PBS, 2 mM, pH 8.0) was chosen as the buffer solution. Mucin from porcine stomach (80 mg, bound sialic acid 0.5%-1.5%) was dissolved in 1 mL PBS buffer to make an 80 mg/mL mucin stock. After brief vortexing, the appropriate volumes of the stock solution of each lipid component and Nile red were pipetted into a clean vial per calculations on a

5 mM, 240 μ L total lipid scale, to obtain the desired molar percentage of each component. As an example, for liposomes composed of 10% **4.6a**, 85% PC, and 5% Nile red, 31.9 μ L PC stock (78.6 μ g), 24 μ L **4.6a** stock (3.8 μ g), and 12 μ L Nile red stock (1.91 μ g) were combined. After 30 seconds of vortexing, the chloroform solvent was dried with a nitrogen stream, and the lipids were subsequently dried for at least 3 h under vacuum. Then, the lipids were hydrated with 240 μ L of PBS buffer, incubated on a 60°C water bath for 3 sets of 20 min, with vortexing after each set. Liposomes then underwent freeze-thaw cycles between a -80°C dry ice-acetone bath and a 60°C water bath for 10 cycles. Vortexing at low speed was performed during each freeze-thaw cycle. Next, liposomes were extruded through a 200 nm membrane filter for 21 passes using a LiposoFast extruder (Avestin, Inc.), and then added to a fresh vial. DLS scans were performed to confirm the formation of stable liposomes. For fluorescence-based release studies, 100 μ L samples of liposome vesicles were added into a sub-micro cuvette cell (Starna Cells, Inc). After an initial fluorescence scan (excitation wavelength of 553 nm, emission wavelength scanned from 560 nm to 580 nm), 0.5 μ L of mucin stock (0.52 U) was added into the cuvette before each fluorescence scan, which was performed 20 times. Data presented in plots indicate emission values at 621 nm. To account for the dilution caused by the addition of heparin solution, blank samples were run by diluting liposomes with the same volumes of water, which were subtracted from the study samples as follows. From the observed fluorescence during the measurement period, a normalized release function that describes the efflux over the period of the experiment was determined

according to the equation below to exclude buffer dilution, where $F(tx)$ and $F(bx)$ are the measured fluorescence at titration times x with mucin and buffer accordingly, and $F(t0)$ and $F(b0)$ are initial fluorescence values for the 2 different sets:

$$\% \text{ release} = \frac{F(tx) - F(bx)}{F(t0) - F(b0)} * 100$$

Sulforhodamine B liposome release studies from bisboronic acid liposomes upon mucin titration

Stock solutions were initiated by weighing out samples of PC and bisboronic acid lipid **4.6a-e** in separate vials to generate separate 32 mM PC and 5 mM **4.6a-e** or **3.5** solutions in ethanol-free chloroform. Phosphate-buffered saline (2 mM, pH 8.0) was chosen as the buffer solution. Mucin from porcine stomach (80 mg, bound sialic acid 0.5%-1.5%) was dissolved in 1mL PBS buffer to make 80 mg/mL mucin stock. Sulforhodamine B sodium salt (116.1 mg) was dissolved in 10 mL PBS buffer to make a 20 mM stock solution. After brief vortexing, proper volumes of each lipid stock solution were pipetted into a clean vial per calculations on a 10 mM, 240 μ L total lipid scale to obtain the desired molar percentage of each component. After 30 seconds of vortexing, the chloroform solvent was dried with a nitrogen stream, and the lipids were subsequently dried for at least 3 h under vacuum. Next, the lipids were hydrated with 240 μ L of the previously described Sulforhodamine B stock solution and incubated on a water bath at 60°C for 3 sets of 20 min, with vortexing after each set. Liposomes then underwent freeze-thaw cycling

between a -80°C dry ice-acetone bath and a 60°C water bath for 10 cycles. Vortexing at low speed was performed during each freeze-thaw cycle. After that, liposomes were extruded through a 200 nm membrane filter for 21 passes using a LiposoFast extruder (Avestin, Inc.) and then added to a fresh vial. Next, liposomes were separated from unencapsulated dye using a Sephadex G-50 size exclusion column (SEC). Liposomes came out in the first or second fraction and were detected by the change in fluorescence upon treatment of an aliquot with triton X-100. DLS scans were performed to confirm the formation of stable liposomes. For fluorescence-based release studies, 100 μ L liposome samples were added into a sub-microcuvette cell (Starna Cells, Inc). After an initial fluorescence scan (excitation wavelength at 553 nm, emission wavelength scanned from 560 nm to 660 nm), 0.5 μ L mucin stock was added for each fluorescence measurement with a time interval of 10 mins before quenching by adding 1 μ L 1% (v/v) Triton-X 100 in PBS to convert liposomes into mixed micelles and trigger rhodamine release. Data presented in plots indicate emission at 606 nm. To account for the dilution caused by the addition of mucin solution, blank samples were run by diluting liposomes with the same volumes of water, which were subtracted from the study samples as follows. From the observed fluorescence during the measurement period, a normalized release function that describes the efflux over the period of the experiment was determined according to the equation below to exclude buffer dilution where $F(t)$, $F(x)$, $F(0)$ are the fluorescence intensity measured after adding Triton X-100, x addition time of mucin stock and initial fluorescence intensity, respectively.:

$$\% \text{ release} = \left(1 - \frac{F(t) - F(x)}{F(t) - F(0)} \right) * 100\%$$

STEM Imaging Studies

Liposome solutions (5mM) containing either 0% or 10% of bisboronic acid lipid **4.6b**, and the remaining portion consisting of PC (100% or 90%, respectively) were prepared using the standard methods described above. One portion of liposomes (100 μ L) was mixed with 10 μ L (800 μ g) porcine mucin, and was then incubated at rt for 30 min, while another aliquot was mixed with 10 μ L PBS buffer solution and also incubated at rt for the same amount of time. After that, both portions were diluted to 400 μ M solutions with PBS buffer to reach ideal concentrations for scanning transmission electron microscopy (STEM) studies. A drop (5–10 μ L) from each solution was immobilized onto separate carbon films supported by 200 mesh copper grids. After 1 minute the grid with sample was washed with distilled water then stained with 1% uranyl acetate for 1 minute then dried. Images were collected using a Zeiss Auriga operating in scanning transmission mode and with a beam energy of 30 KeV.

Cell culture and fluorescence microscopy studies of cell delivery using bisboronic acid

4.6b

Melanoma A375 cells, obtained from ATCC[®] (Manassas, VA), were cultured in a humidified incubator under 5% CO₂ in DMEM medium supplemented with 10% of Fetal Bovine Serum, 50 U/mL penicillin, and 50 µg/ml streptomycin (Invitrogen). Two days prior to the experiment, A375 cells were plated at a seeding density of 1×10^4 cells per well on a glass bottom 96 well plate. Liposomes containing 0.08% rhodamine L- α -phosphatidylethanolamine (Rd-PE) as well as 0%, 2%, 5% or 10% of bisboronic acid lipid **4.6b**, with the rest of the liposome consisting of PC, were incubated with cells at a concentration of 1 mM for 2h at 37°C. Liposome preparation was conducted under standard conditions, as described above, using 10mM PBS pH=7.4 as the buffer. After incubation, cells were washed four times with PBS containing 1mM MgCl₂ and 100mM CaCl₂ (PBS⁺⁺), fixed for 30 min in 4% paraformaldehyde and the nucleus was stained with DAPI (Invitrogen). Images were acquired on Cytation 5 plate reader (Biotek) with a 20x lenses. Contrast and brightness settings were chosen so that all pixels were in the linear range.

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Appendices

Appendix A Figures and tables

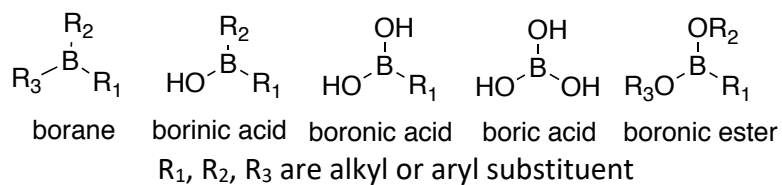


Figure 1.1. Name and structure of oxygen-containing organoboron compounds.

The nomenclature of organoboron compounds is based on the substituents bonded to boron atom.

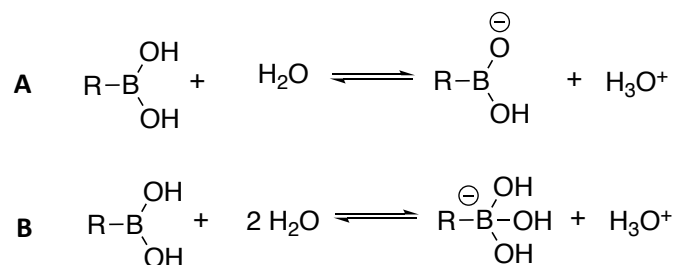


Figure 1.2. Ionization equilibrium of boronic acids in water.

In the first equilibrium (A), the boronic acid follows Brønsted–Lowry theory and the conjugate base is generated through exchange of a proton. In the second equilibrium (B), the boronic acid acts as a Lewis acid by accepting a pair of electrons from an oxygen nucleophile to generate the conjugated base. It has been proven that boronic acids show more Lewis acidity due to the vacant p orbital on boron.

Table 1.1. Ionization constant (pK_a) for selected boronic acids.

Data courtesy of review by Dennis Hall[1]

Boronic acid, $RB(OH)_2$	pK_a	Reference
Boric acid, $B(OH)_3$	9.0	[3]
Methyl	10.4	[3]
Phenyl	8.9	[4]
3,5-Dichlorophenyl	7.4	[4]
3,5-Bis(trifluoromethyl)phenyl	7.2	[4]
2-Methoxyphenyl	9.0	[5]
3-Methoxyphenyl	8.7	[4]
4-Methoxyphenyl	9.3	[6]
4-Carboxyphenyl	8.4	[7]
2-Nitrophenyl	9.2	[8]
4-Nitrophenyl	7.1	[6]
4-Bromophenyl	8.6	[4]
4-Fluorophenyl	9.1	[4]
2-Methylphenyl	9.7	[9]
3-Methylphenyl	9.0	[9]
4-Methylphenyl	9.3	[9]
3,5-Dimethylphenyl	9.1	[4]
3-Methoxycarbonyl-5-nitrophenyl	6.9	[11]
2-Fluoro-5-nitrophenyl	6.0	[5]
3-Pyridyl	4.0, 8.2	[5]
3-Benzyl-3-pyridylum	4.2	[5]
Quinolinyl	4.0, 10	[10]

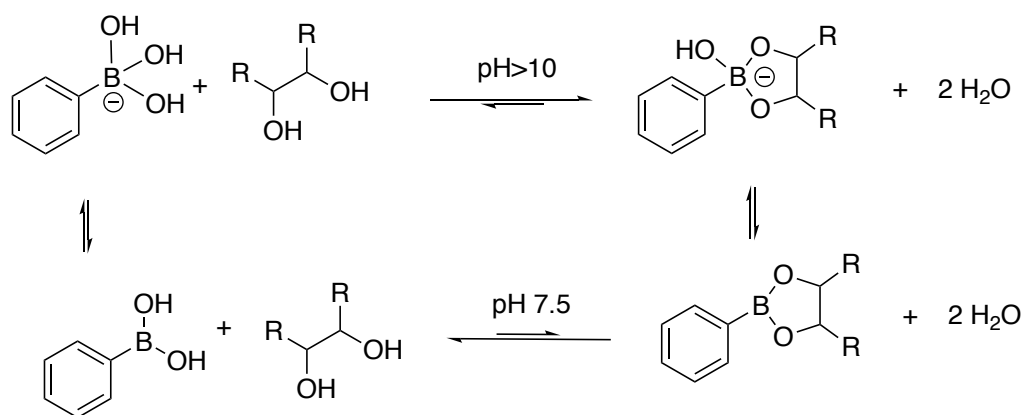


Figure 1.3 Equilibrium for formation of boronate esters from diols at high and neutral pH in aqueous solution.

Higher pH generally favors the formation of boronate esters from boronic acids and diols because elevated hydroxide concentrations ensure the boronate ester is “locked” in the more stable tetrahedral form.

Table 1.2. Association constants (K_{eq}) of phenylboronic acid with diols.

Data courtesy of paper reported by Wang and coworkers.[65]

Diol	K_{eq} (M^{-1})	Diol	K_{eq} (M^{-1})
Alizarin Red S.	1300	Sialic acid	21
Catechol	830	<i>cis</i> -1,2-Cyclopentane diol	20
D-sorbitol	370	Glucuronic acid	16
D-fructose	160	D-galactose	15
D-tagatose	130	D-xylose	14
D-mannitol	120	D-mannose	13
L-sorbose	120	D-glucose	4.6
1,4-Anhydroerythritol	110	Diethyl tartrate	3.7
D-erythronic- γ -lactone	30	Maltose	2.5
L-arabinose	25	Lactose	1.6
D-ribose	24	Sucrose	0.67

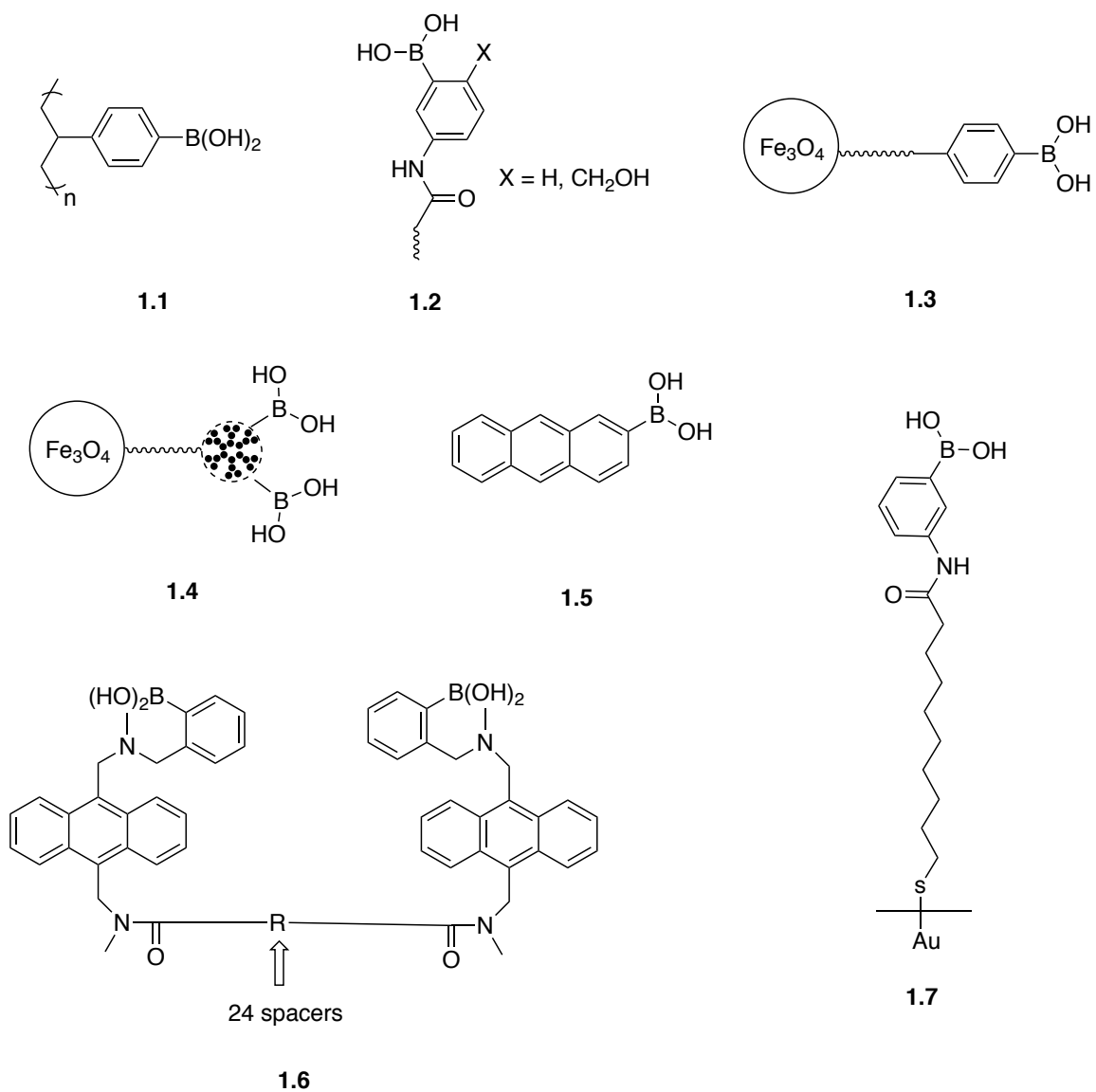
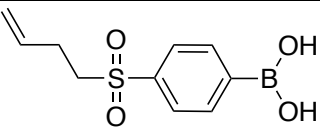
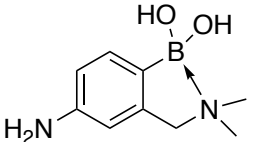
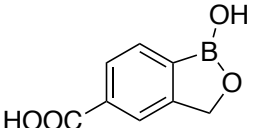
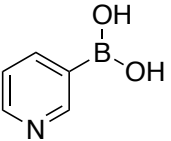
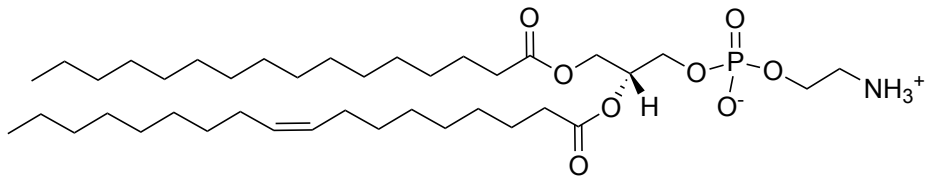


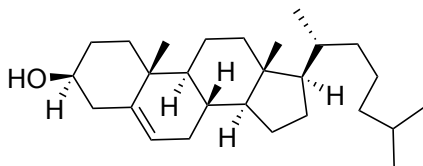
Figure 1.4. Structures of some representative boronic acid materials for carbohydrate recognition and enrichment.

Table 1.3. Structure and pK_a of representative boronate affinity materials.

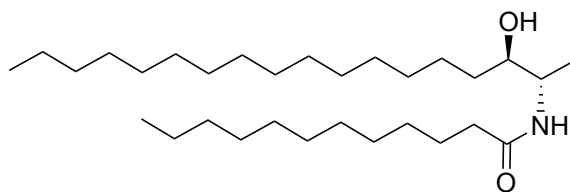
Type	Representative structures	pK_a
Boronic acid with electron-withdrawing groups		7.0[34]
Wulff-type boronic acid		5.2[12]
Improved Wulff-type boronic acid (Benzoboroxoles)		6.9[162]
Heterocyclic boronic acid		4.4[75]



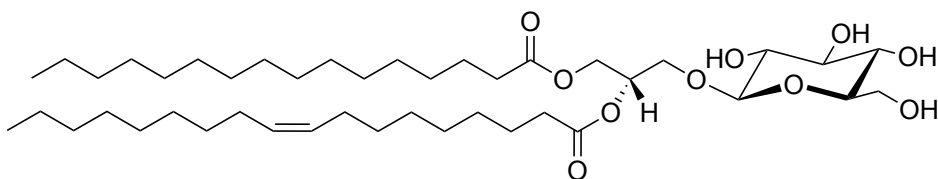
Phospholipids :1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine



Sterol Lipids: cholest-5-en-3 β -ol



Sphingolipids: N-dodecanoyl-1-deoxysphinganine



Glycolipids: 1,2-diacyl-3-O-(α -D-glucopyranosyl)-*sn*-glycerol

Figure 1.5 Representative structures of different types of complex lipids

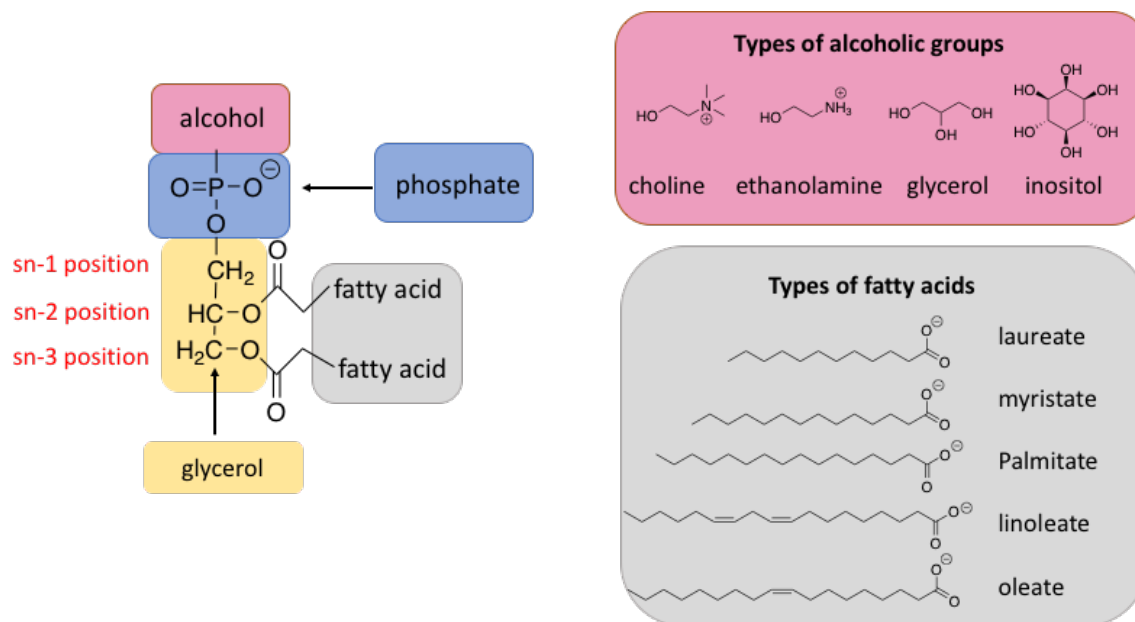
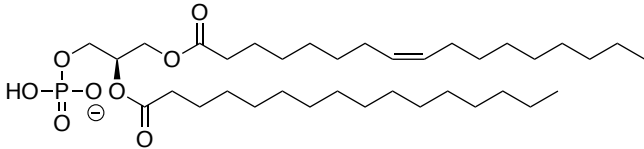
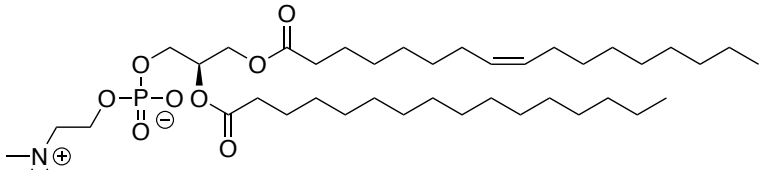
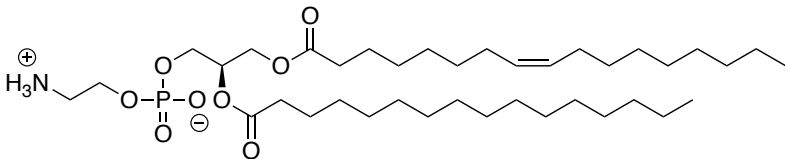
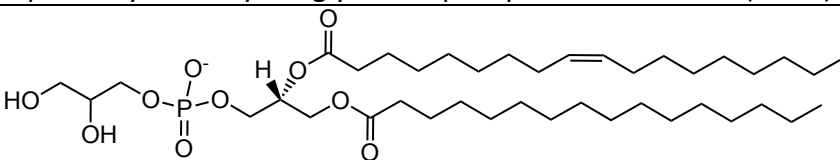
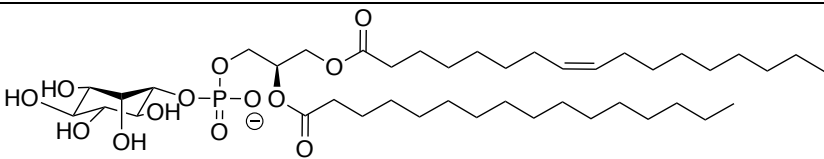
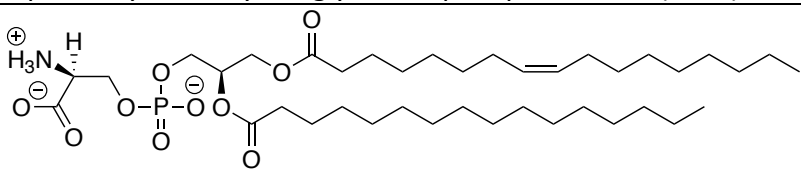


Figure 1.6. General Structure of phosphoglycerides courtesy of book chapter by Strasser and Wittmann.[85]

The common structural features of phosphoglycerides include glycerol backbones, phosphodiester headgroups and one or two acyl chain(s). The types of phosphoglycerides depend on the alcohol groups bonded to phosphate and hydrophobic fatty acids chains.

Table 1.4. Nomenclature, headgroups, structures of common representative phosphoglycerides

Name	Headgroup	Structure of representative lipids
Phosphatidic acid (PA)	none	 1-Oleoyl-2-palmitoyl-phosphatidic acid (POPA)
Phosphatidylcholine (PC)	choline	 1-Oleoyl-2-palmitoyl-phosphatidylcholine (POPC)
Phosphatidylethanolamine (PE)	ethanolamine	 1-palmitoyl-2-oleoyl-<i>sn</i>-glycero-3-phosphoethanolamine (POPE)
Phosphatidylglycerol (PG)	glycerol	 1-palmitoyl-2-oleoyl-<i>sn</i>-glycero-3-phospho-(1'-rac-glycerol) (POPG)
Phosphatidylinositol (PI)	inositol	 1-palmitoyl-2-oleoyl-<i>sn</i>-glycero-3-phosphoinositol (POPI)
Phosphatidylserine (PS)	serine	 1-palmitoyl-2-oleoyl-<i>sn</i>-glycero-3-phospho-L-serine (POPS)

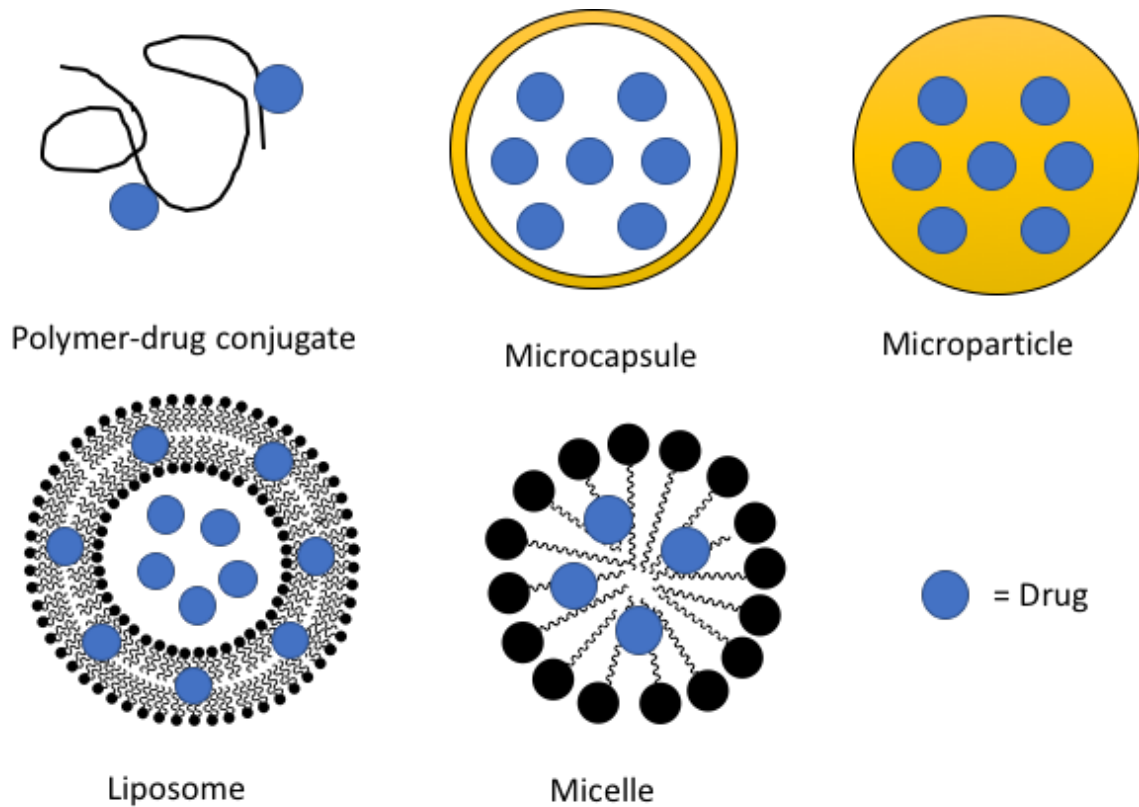


Figure 1.7. Types of drug delivery vesicles.

Several types of drug delivery vesicles have been intensively studied for their ability to bind and recognize the targeted pathological area, including synthetic soluble polymers, microcapsules, microparticles, cells, cell ghosts, liposomes, and micelles.

Table 1.5. Packing parameters of representative lipids and their vesicle formation.

Packing parameter is calculated based on the lipid shape and structure. It can help to determine the optimal shape of the product produced by lipid self-assembly. For example, lyso-phosphatidylcholine (LPC) has a large headgroup to tail ratio, which leads to the formation of micelles. On the other hand, due to the lack of methyl substituent, the headgroup of phosphatidylethanolamine (PE) is smaller compared to tail, which leads to the formation of an inverted cone shape hexagonal structure. Cylindrical lipids such as phosphatidylcholine (PC) instead prefer the formation of membrane bilayers.

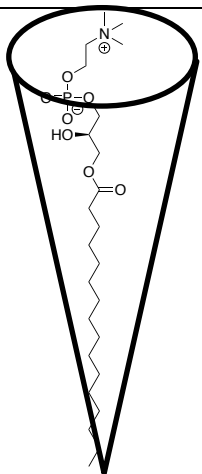
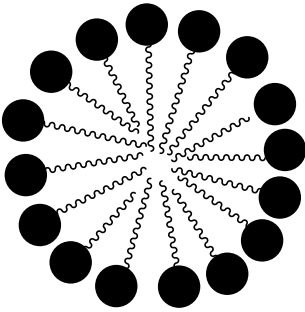
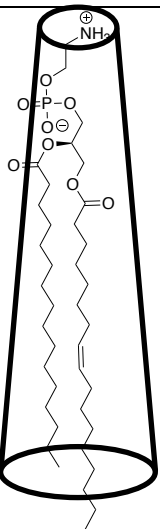
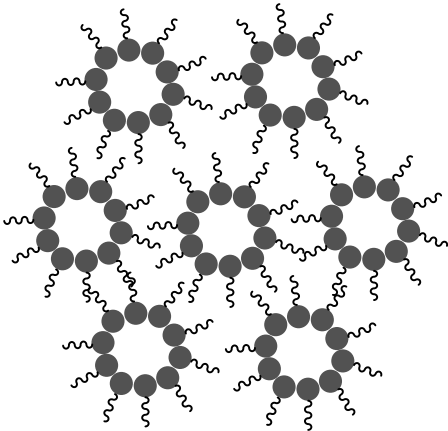
Packing parameter (ρ)	Lipid structure	Shape of assembly
$< 1/3$	 LPC	 Micelle
$1/2 - 1/3$	 PE	 Inverted Hexagonal

Table 1.5. Packing parameters of representative lipids and their vesicle formation.
(continued)

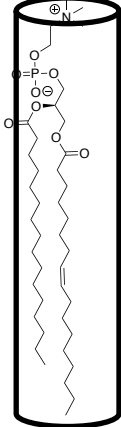
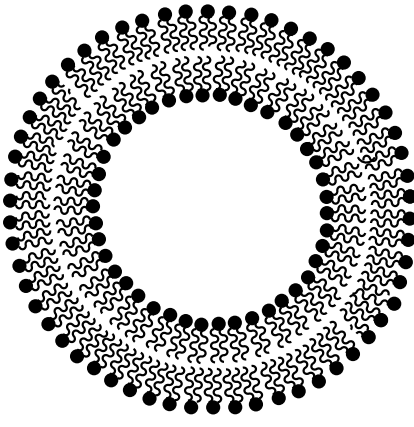
Packing parameter (ρ)	Lipid structure	Shape of assembly
1/2 to 1		
	PC	Bilayers

Table 1.6. List of representative phospholipids and their transition temperatures.

Data courtesy of Avanti Polar Lipids, Inc. website. The transition temperature of phospholipids, which is defined as the temperature where lipids transform from crystal to gel phase, is proportional to length of acyl chain and is inversely proportional to degree of saturation.

Name of phospholipid	T _m (°C)
12:0 Dilauroyl phosphatidylcholine (DLPC)	-2
14:0 Dimyristoyl phosphatidylcholine (DMPC)	24
16:0 Dipalmitoyl phosphatidylcholine (DPPC)	41
18:0 Distearoyl phosphatidylcholine (DSPC)	55
18:1 Dioleoyl phosphatidylcholine (DOPC)	-17
18:0 Distearoyl phosphatidylethanolamine (DSPE)	74
18:1 Dioleoyl phosphatidylethanolamine (DOPE)	-16
18:0 Distearoyl phosphatidylserine (DSPS)	68
18:1 Dioleoyl phosphatidylserine (DOPS)	-11

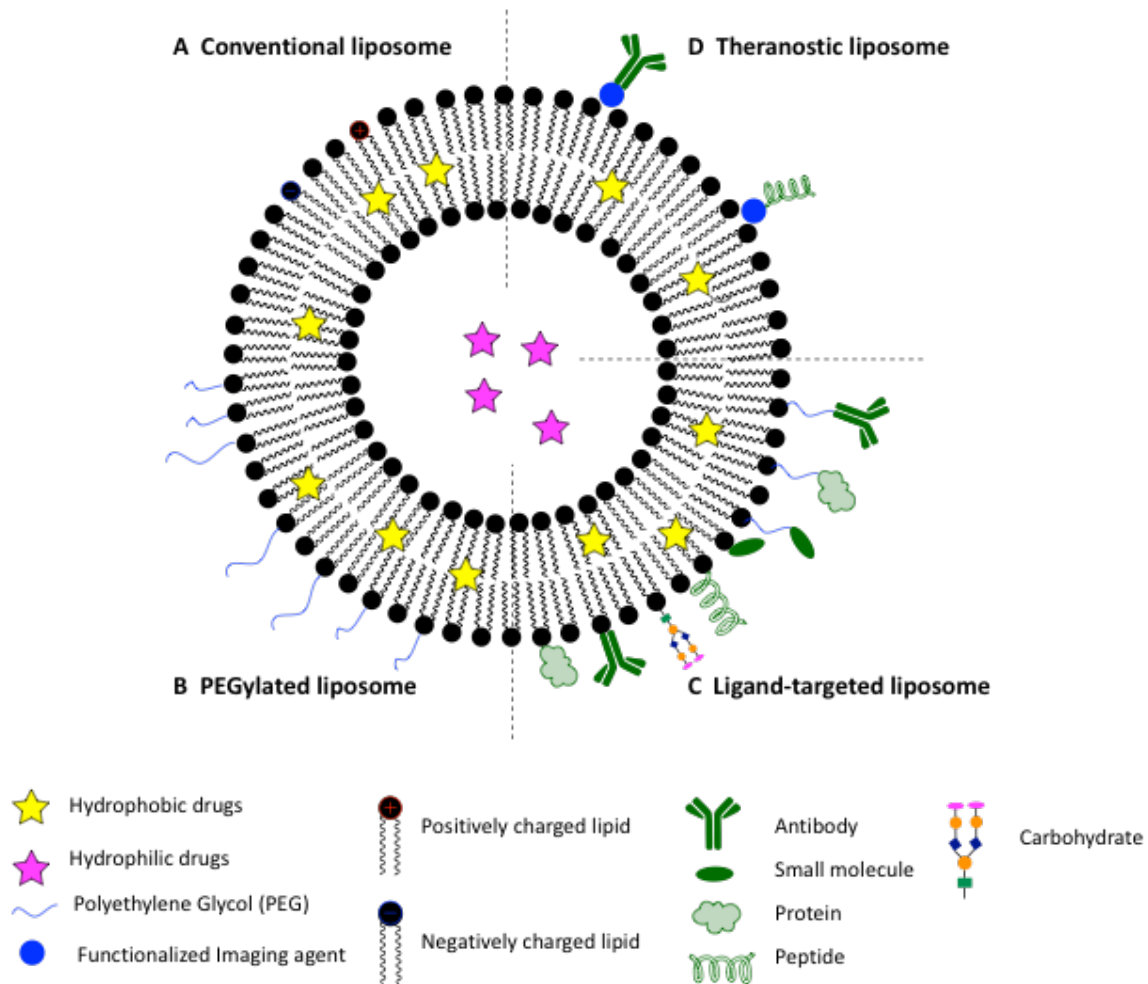


Figure 1.8. Different types of liposomal drug delivery systems.

Scheme inspired by Hua's work.[137] (A) conventional liposomes are composed of cationic, anionic, or neutral phospholipids and cholesterol with lipid bilayers and aqueous core. (B) PEGylated liposomes are decorated with polyethylene glycol (PEG) as a hydrophilic coating to enhance steric stability. (C) Ligand-targeted liposomes are modified with ligands, such as antibodies, peptides, and carbohydrates, for targeting specific cell types (D) Theranostic liposomes are combinations of the three types above with nanoparticles, targeting elements, imaging components and therapeutic components

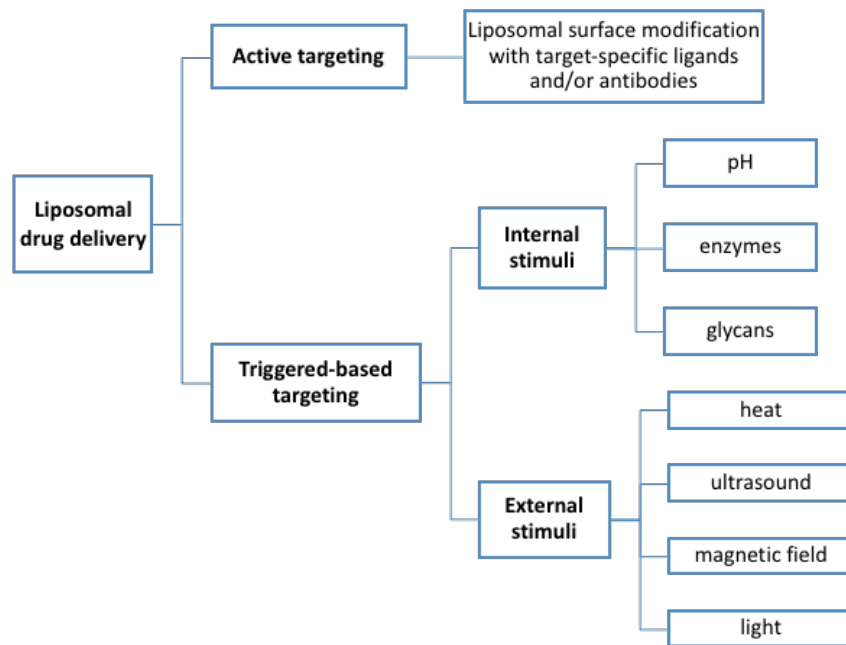


Figure 1.9. Different type of liposome drug delivery method.

Liposomal drug delivery can generally be categorized into active targeting, which can be achieved by liposomal surface decoration with target-specific ligands and/or antibodies, and triggered-based targeting, which utilizes internal stimuli, such as pH or enzymes, and external stimuli, such as ultrasound, heat, light, magnetic field

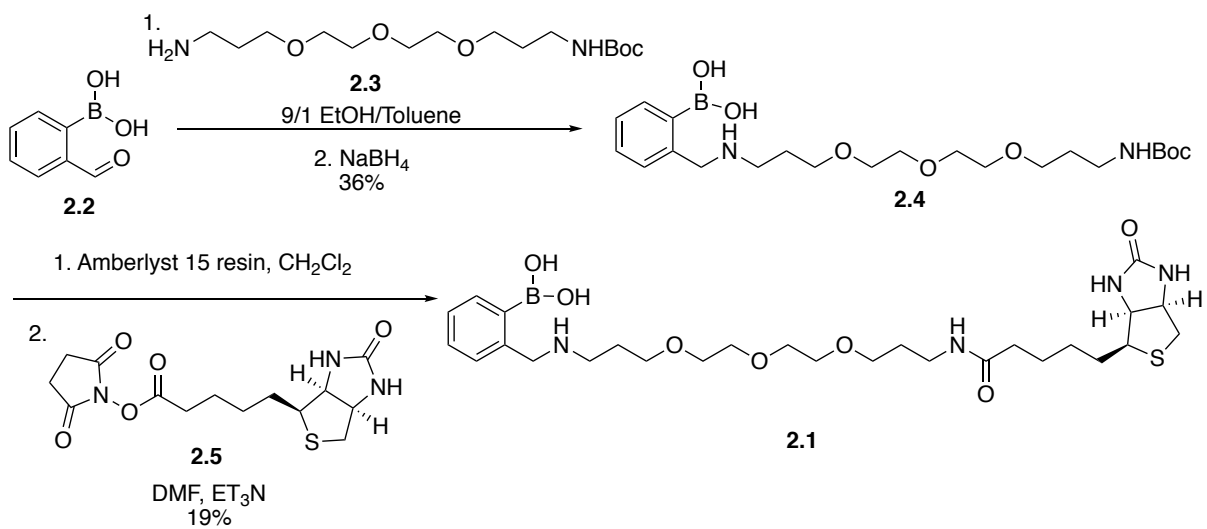


Figure 2.1. Synthetic route to boronic acid-biotin conjugate 2.1.

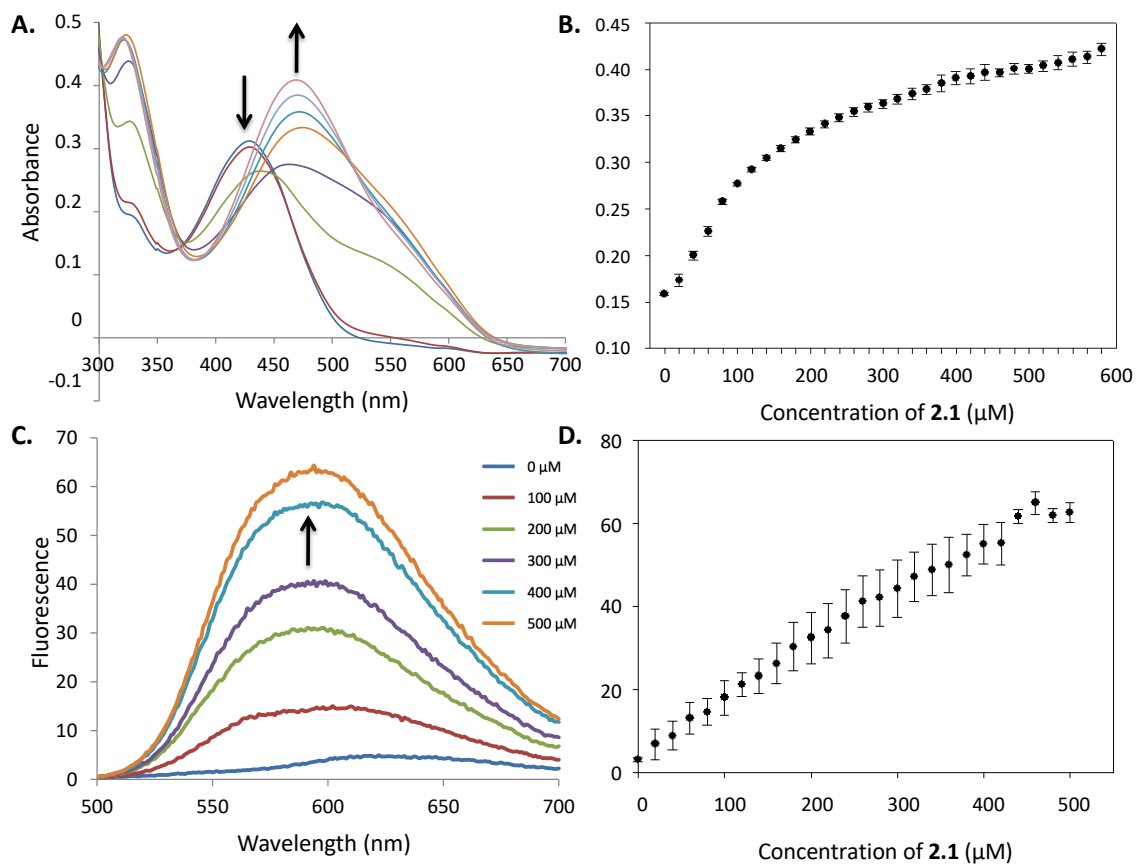


Figure 2.2. Data from UV/vis and fluorescence titrations of 2.1 into alizarin.

A. Spectral overlay of UV/vis shift with selected points from 0-600 μM 2.1. B. Plot of absorbance increase. C. Spectral overlay of fluorescence increase. D. Plot of fluorescence increase with selected points from 0-500 μM 2.1. Data are presented as the average of at least three experiments with error bars depicting standard error.

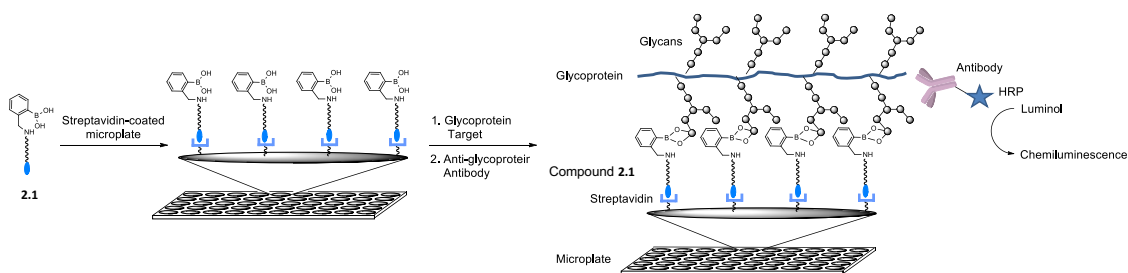


Figure 2.3. Illustration of boronic acid assay and glycoprotein detection.

Boronic acid–biotin conjugate 2.1 is immobilized onto streptavidin microplates creating a multivalent sensor surface. Incubated glycoproteins can then bind to this surface through boronic ester formation, and chemiluminescence detection of bound protein can be performed without (HRP) or with (mucin-1) the use of an HRP-tagged antibody.

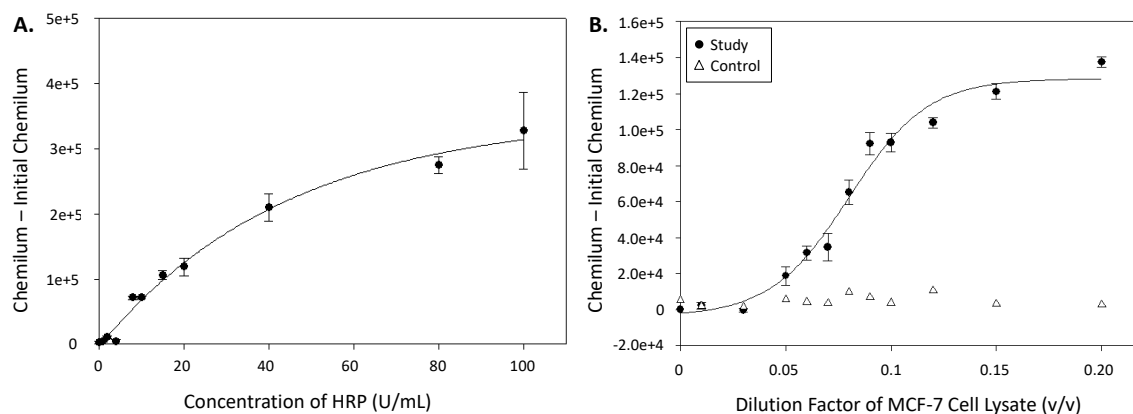


Figure 2.4. Data from chemiluminescence detection of glycoprotein binding using boronic acid microplate assay.

A. Detection of purified HRP protein binding. B. Detection of Muc1 from MCF-7 cancer cell lysates. Data are presented as the average of at least three experiments with error bars depicting standard error.

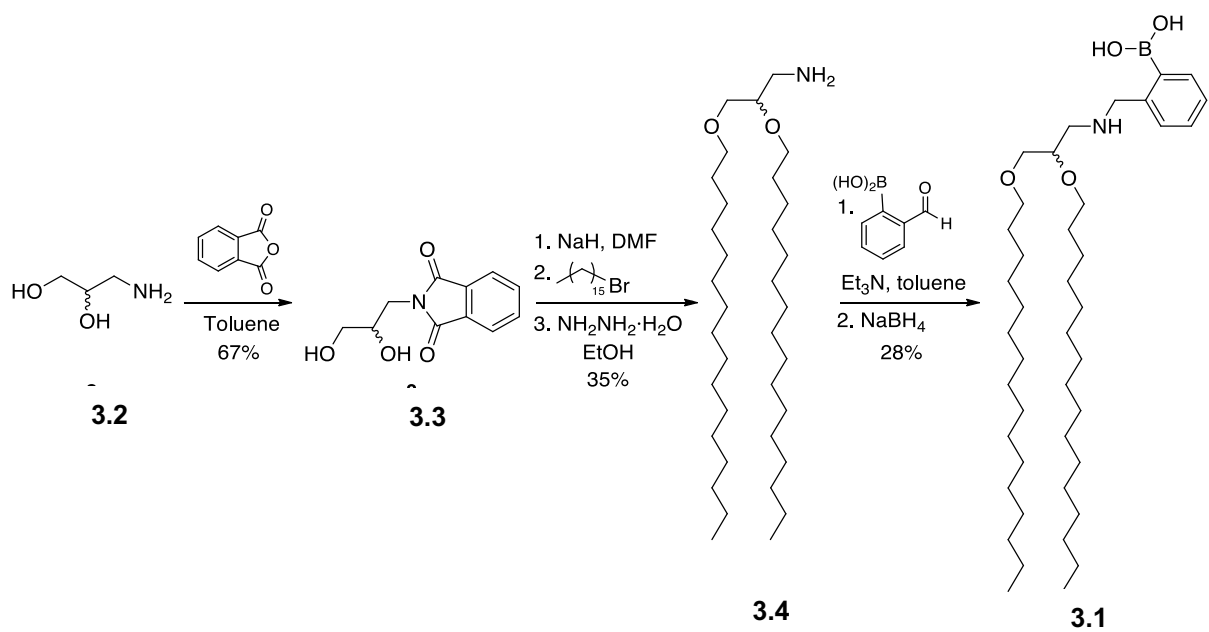


Figure 3.1. Synthetic route for boronic acid lipid **3.1**.

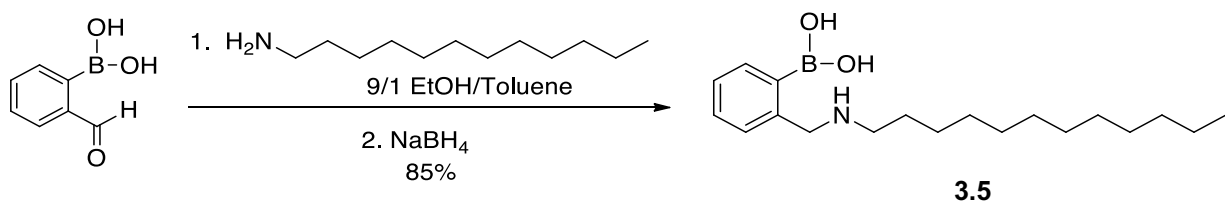


Figure 3.2. Synthetic route for boronic acid lipid 3.5.

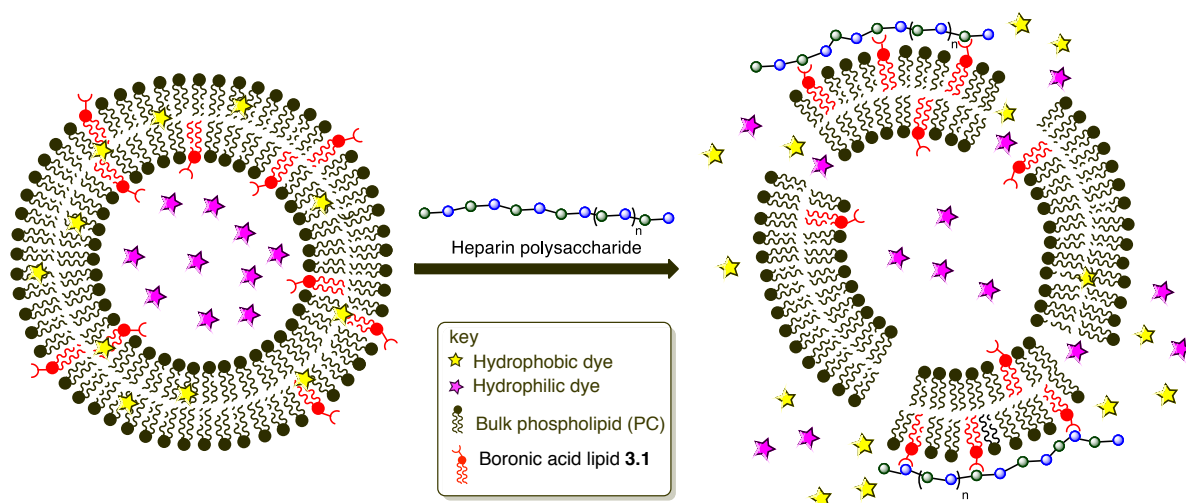


Figure 3.3 . Fluorescence-based dye leakage assays driven by heparin-boronic acid lipid interactions.

Hydrophobic (Nile red) or hydrophilic (sulforhodamine B) dyes are separately encapsulated within boronic acid liposome membrane bilayers or aqueous interiors, respectively. Heparin carbohydrate binding is evaluated for the release of contents leading to fluorescence decreases (Nile red) or increases (sulforhodamine B) attributed to membrane distortion upon carbohydrate binding.

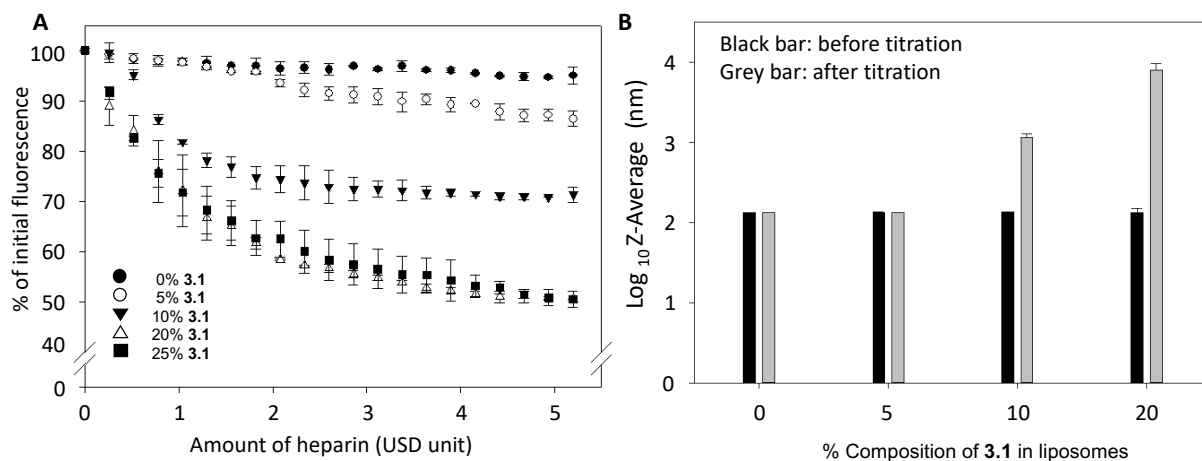


Figure 3.4. Boronic acid liposome Nile red release and DLS results upon heparin incubation.

A. Decreases in Nile red fluorescence attributed to release are dependent upon the percentage of boronic acid lipid 3.1 in PC liposomes. B. DLS results show that untreated liposomes exhibit the expected sizes, while treatment of liposomes containing higher percentages (10-20%) of 3.1 with heparin leads to much larger particle sizes attributed to lipid reorganization. Error bars denote the standard errors of at least three replicates.

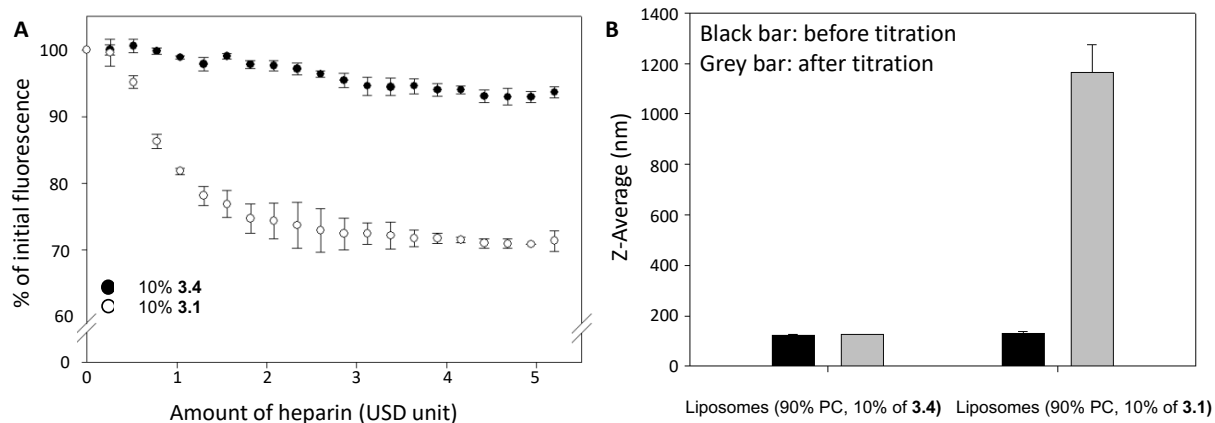


Figure 3.5. Nile red release and DLS results upon heparin incubation using control compound 3.4.

A. Decreases in Nile red fluorescence were much greater for PC liposomes containing 10% of boronic acid lipid 3.1 compared to those instead containing 10% of control compound 3.4. B. DLS results show that liposomes containing 3.1 exhibit large increases size upon heparin treatment while those instead containing compound 3.4 do not.

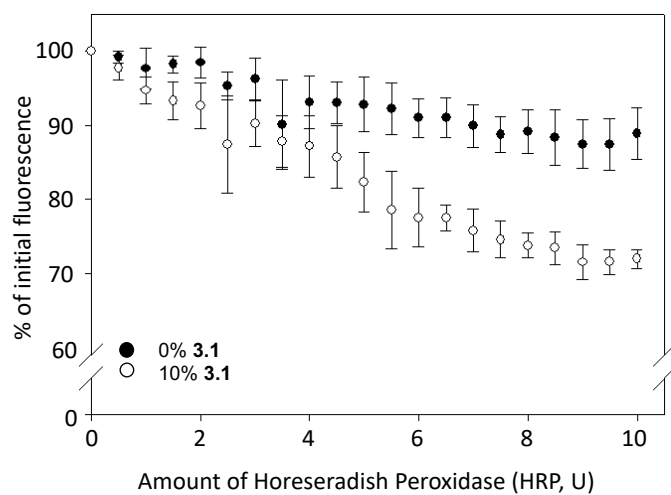


Figure 3.6. Nile red release titration of liposomes containing 3.1 with HRP glycoprotein.

Decreases in Nile red fluorescence were greater for PC liposomes containing 10% of boronic acid lipid 3.1 compared to those PC liposomes lacking 3.1.

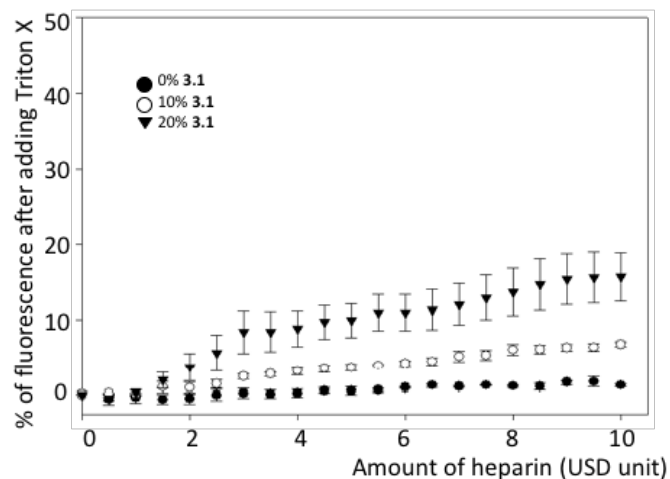


Figure 3.7. Boronic acid liposome sulforhodamine release results upon heparin titration.

Fluorescence increases upon sulforhodamine B release, plotted as a percentage of increases caused by Triton X treatment, are shown to correlate with the percentage of 3.1 in PC liposomes. Error bars denote the standard errors of at least three replicates.

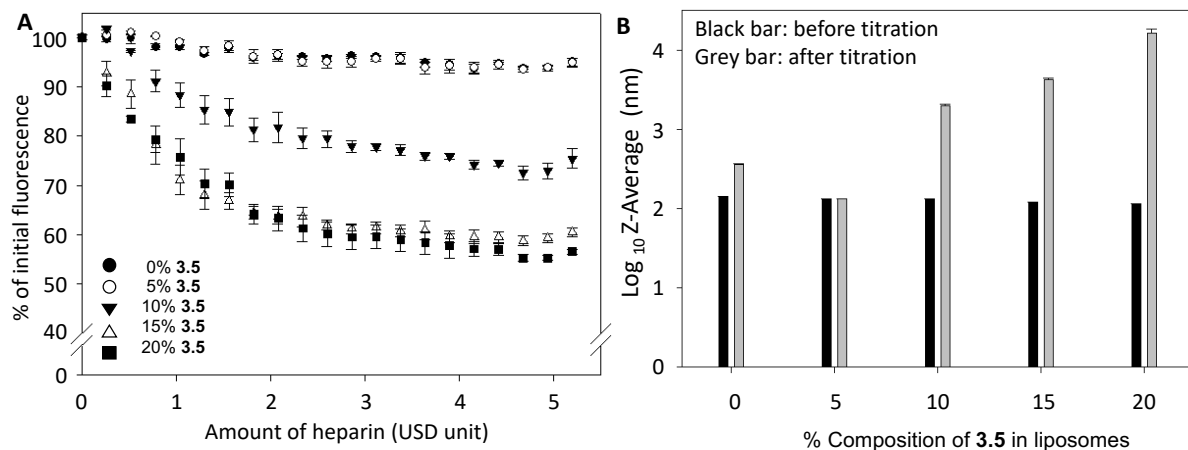


Figure 3.8. Boronic acid liposome Nile red release and DLS results upon heparin incubation using compound 3.5.

A. Decreases in Nile red fluorescence attributed to release are dependent upon the percentage of boronic acid lipid 3.5 in PC liposomes. **B.** DLS results show that untreated liposomes exhibit the expected sizes, while treatment of liposomes containing higher percentages (10-20%) of 3.5 with heparin leads to much larger particle sizes. Error bars denote the standard errors of at least three replicates.

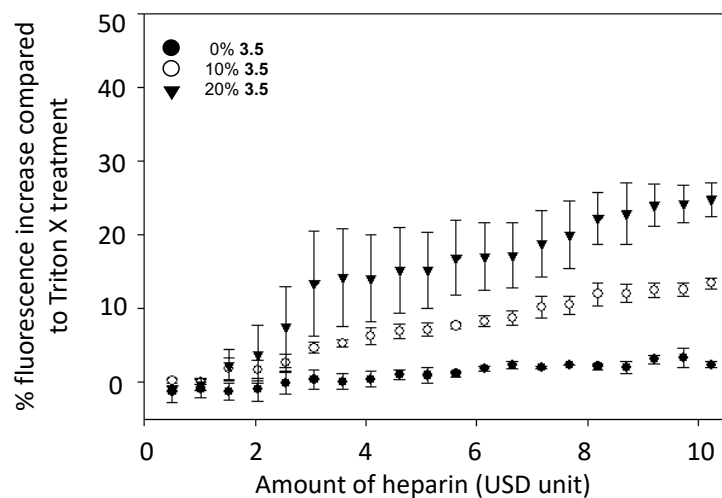


Figure 3.9. Boronic acid liposome sulforhodamine release upon heparin titration using 3.5.

A. Fluorescence increases upon sulforhodamine. B release are shown to correlate with the percentage of 3.5 in PC liposomes. Error bars denote the standard errors of at least three replicates.

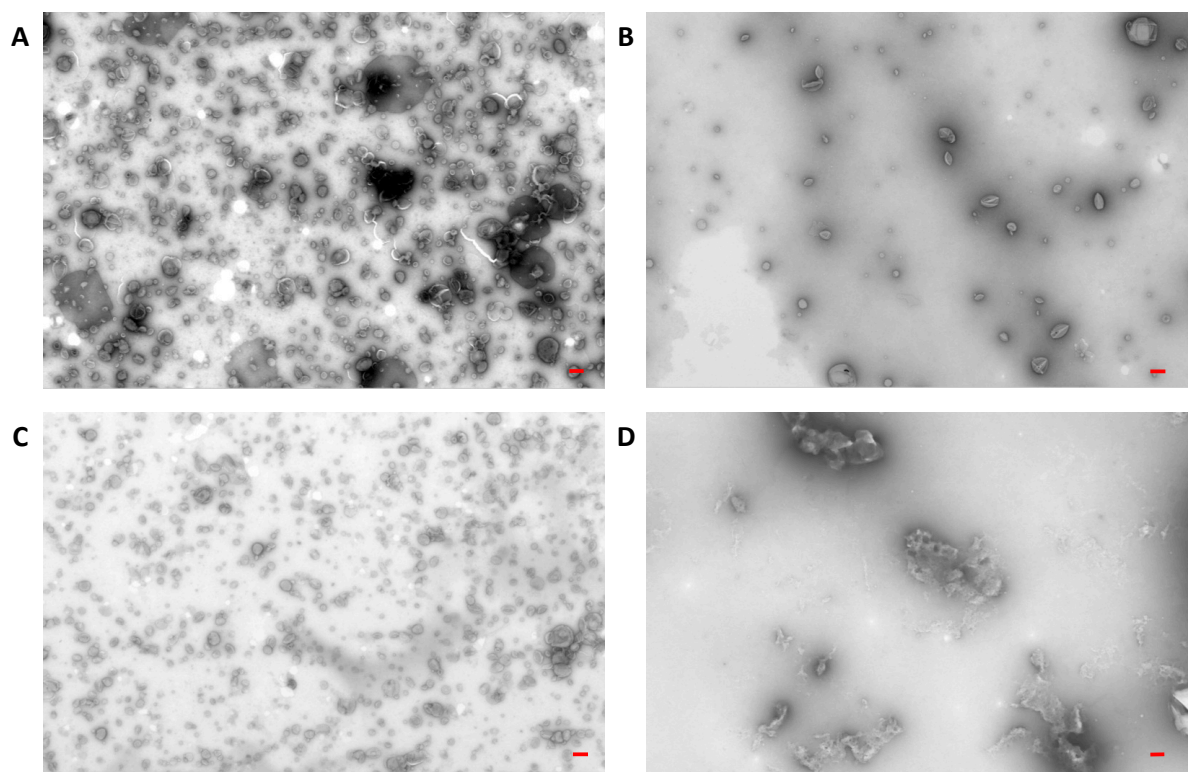


Figure 3.10. STEM Images.

Images are shown for liposomes containing 0% of 3.1 without (A) and with (B) heparin treatment as well as those containing 10% of 3.1 without (C) and with (D) heparin treatment. Scale bars correlate with 200 nm.

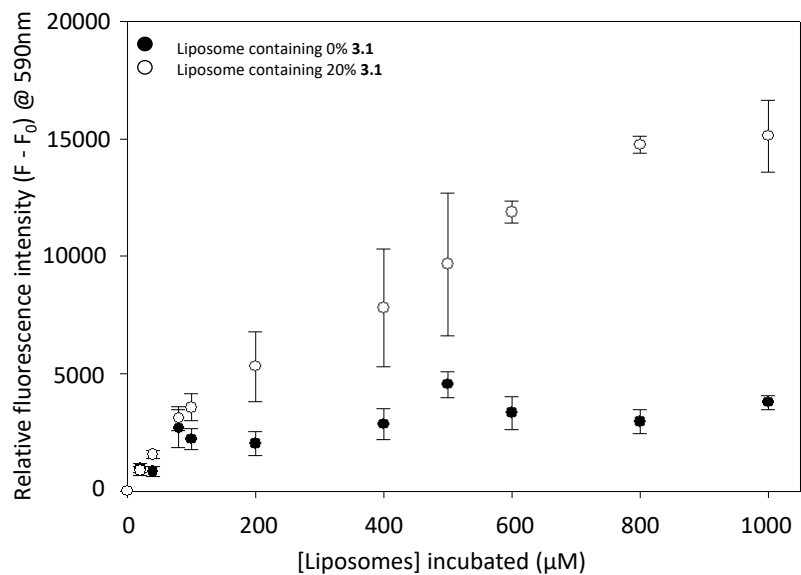


Figure 3.11. Results from microplate studies indicating the binding of fluorescent liposomes to immobilized heparin-biotin.

Error bars indicate standard errors for at least three replicates.

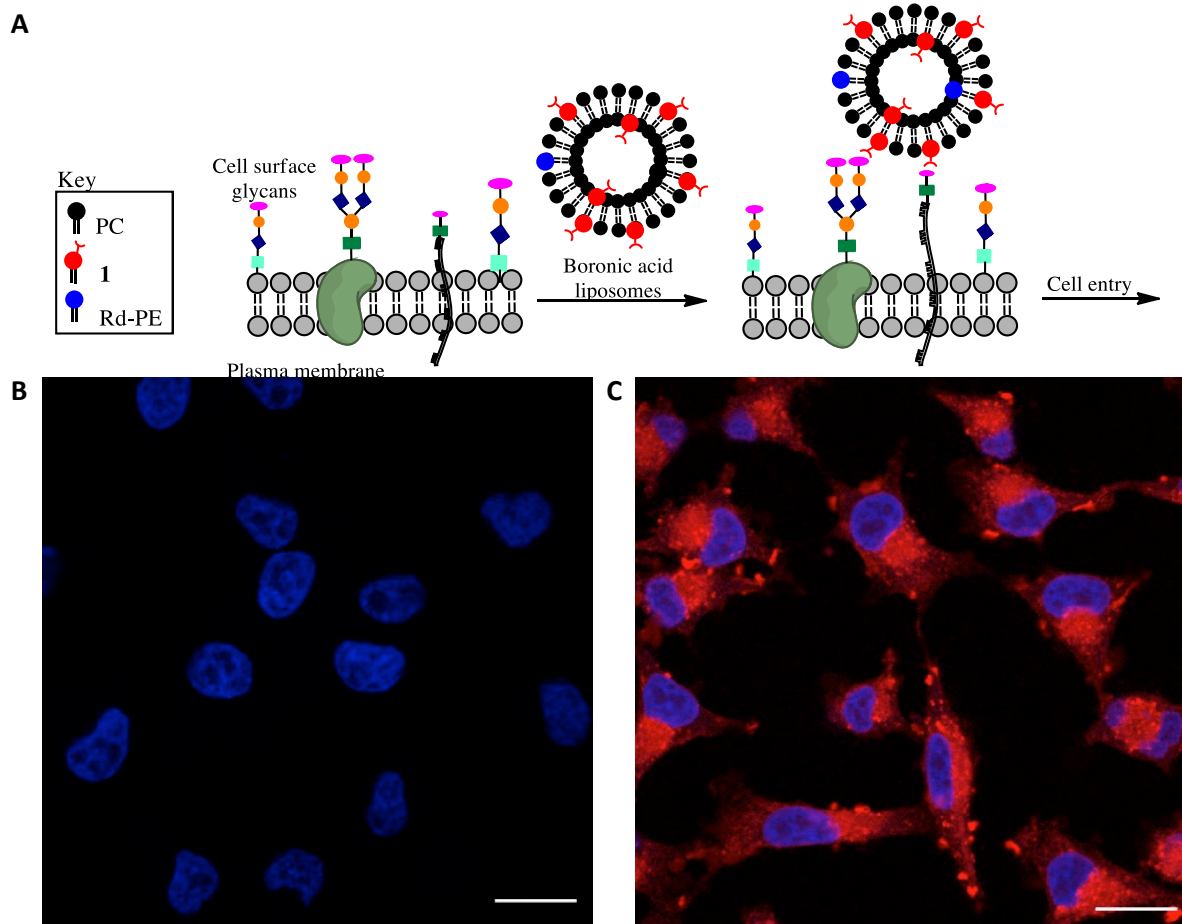


Figure 3.12. Cellular delivery studies.

A. Cartoon depicting liposome cell entry driven by binding interactions with cell surface carbohydrates. **B.** Fluorescence image of cells treated with PC/Rd-PE liposomes. **C.** Image upon treatment with PC/3.1(10%)/Rd-PE liposomes. Scale bar depicts 20 μ M. Fluorescence resulting from DAPI is shown in blue. Fluorescence resulting from Rd-PE is shown in red.

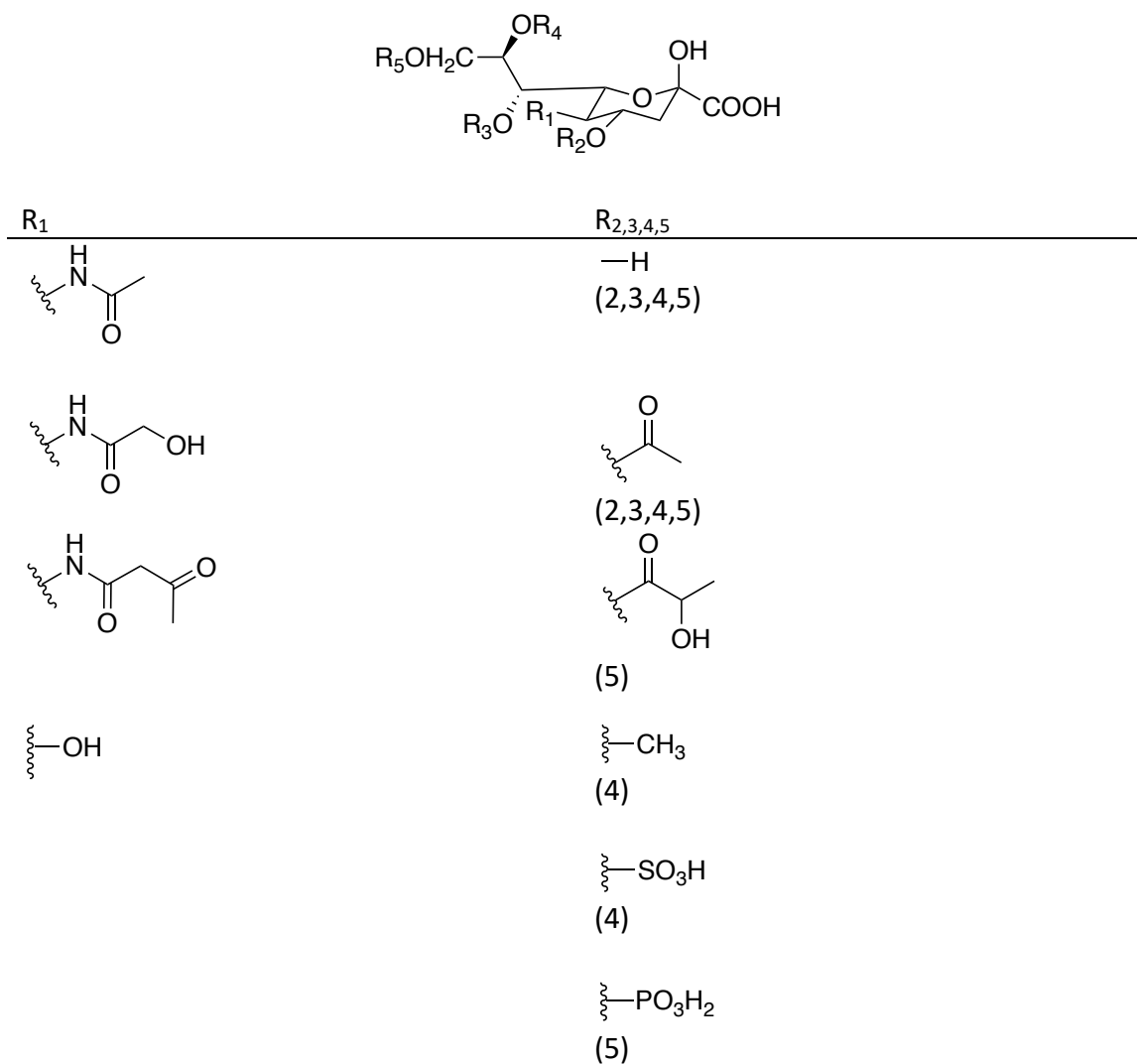
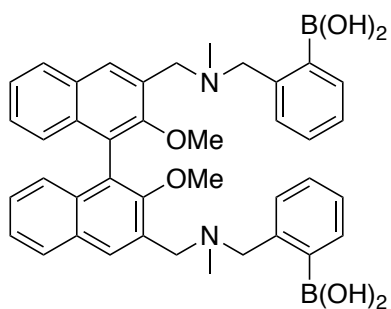
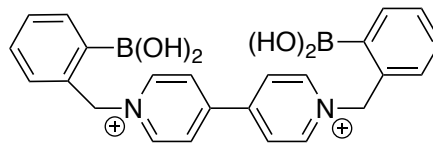


Figure 4.1. Selected structures from the family of sialic acids with a list of natural substituents.

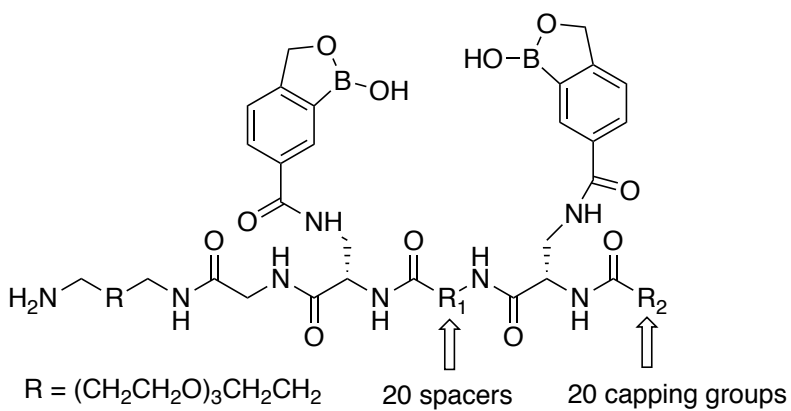
The majority of sialic acids are derivatives of neuraminic acid, such as *N*-acetylneuraminic acid (Neu5Ac) or *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac₂)



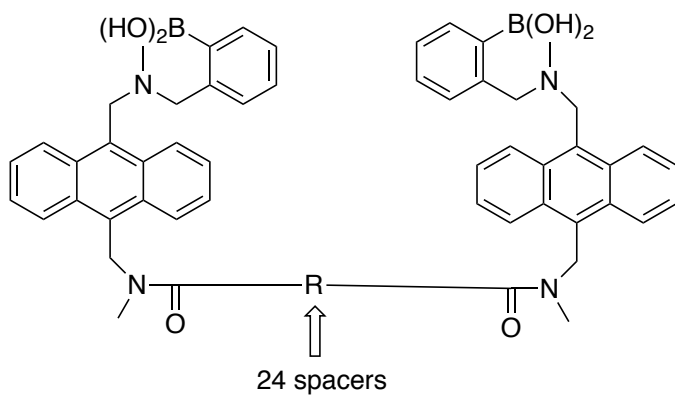
4.1



4.2



4.3



4.4

Figure 4.2. Structures of boronic acid-based sensors for selective recognition of carbohydrates.

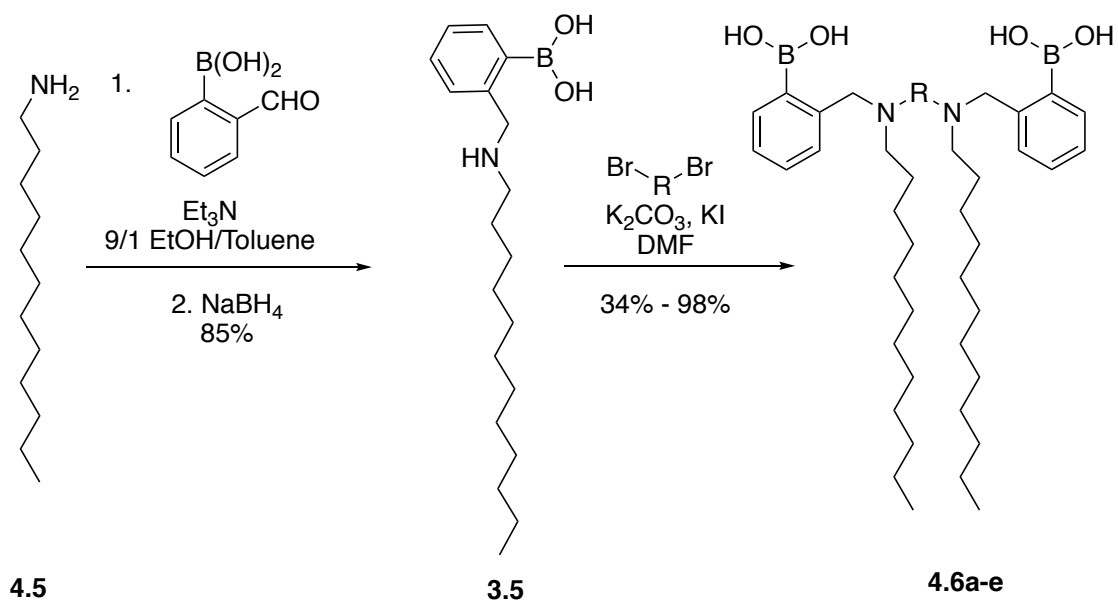
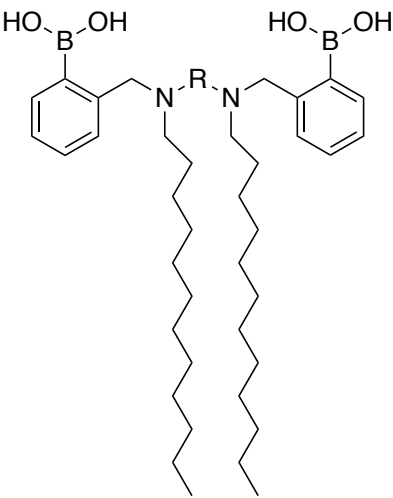
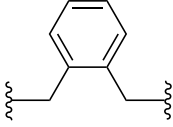
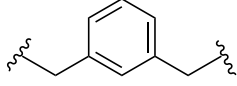
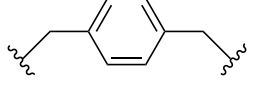
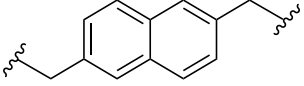
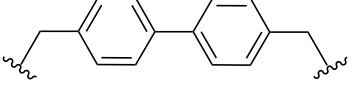


Figure 4.3. Synthetic route for bisboronic acid lipids

Table 4.1. Chemical structures of bisboronic acid lipids 4.6a-e

	
Compounds	R
4.6a	
4.6b	
4.6c	
4.6d	
4.6e	

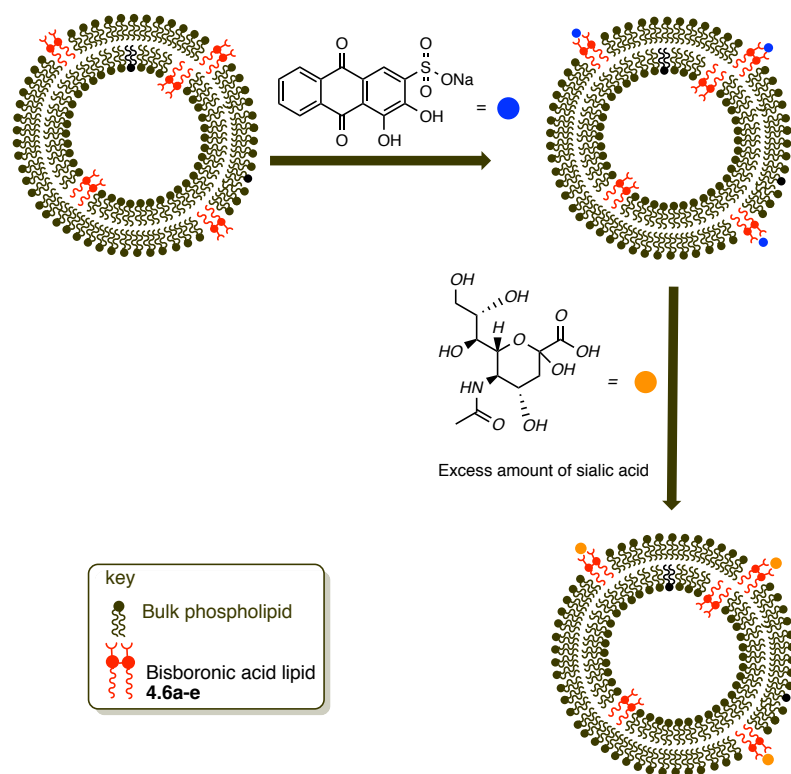


Figure 4.4. Three-component fluorescent competition assay with Alizarin Red S (ARS).

Liposomes incorporated with bisboronic acid lipids 4.6a-z were treated with ARS to reach maximum fluorescence intensity. Then, *N*-Acetylneuraminic acid was titrated into the system to reach an excess amount. Due to the introduction of Neu5Ac, some of the bound ARS was released into the environment, which led to a decrease in fluorescence intensity. The binding affinity of Neu5Ac can then be estimated through data collected from titration kinetics curves.

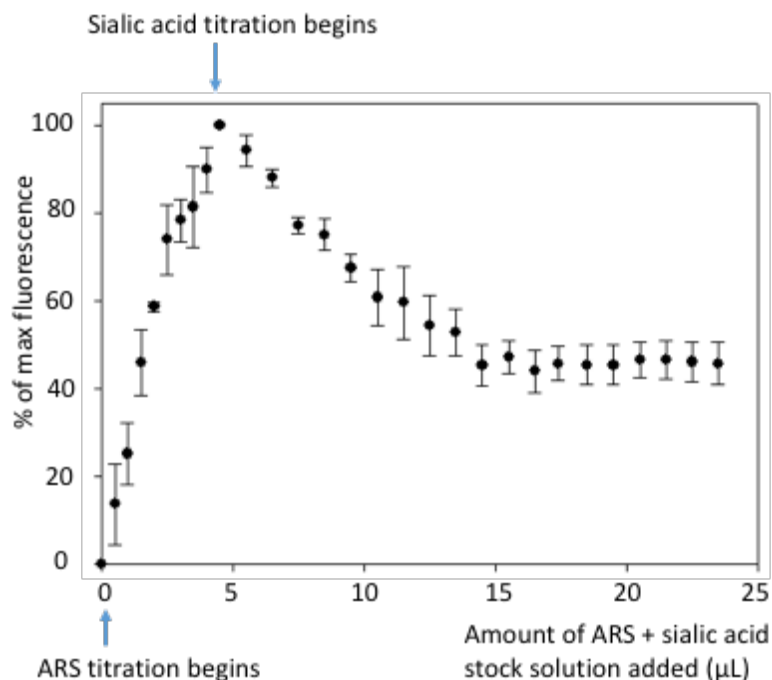


Figure 4.5. Fluorescence titration curve of liposomes containing bisboronic acid lipid 4.6b resulting from an ARS competition assay to probe Neu5Ac binding.

Titration with ARS led to a gradual increase in fluorescence due to boronate formation that caused the removal of active protons from the ARS catechol moiety. Neu5Ac was then titrated in, which eventually led to a decrease of almost 50% of the maximal signal, after which the signal plateaued. Error bars denote the standard errors of at least three replicates.

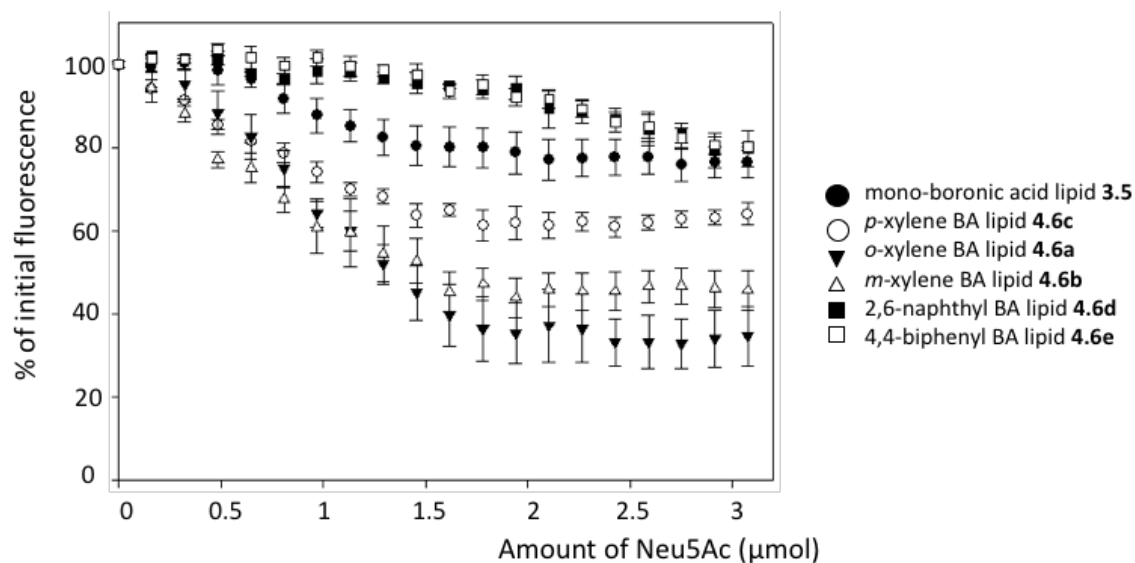


Figure 4.6 Fluorescence titration curves of liposomes containing bisboronic acid lipids 4.6a-e upon treatment with ARS and then competition with Neu5Ac.

First, ARS solution was added to PC liposomes containing 4.6a-e leading to high fluorescence intensity. Then, Neu5Ac was titrated in, resulting in a varied degree of fluorescence decrease, in which liposomes with compound 4.6a had the most significant decrease (~70%) and those with compound 4.6d had the least decrease (~20%) in fluorescence intensity. Error bars denote the standard errors of at least three replicates.

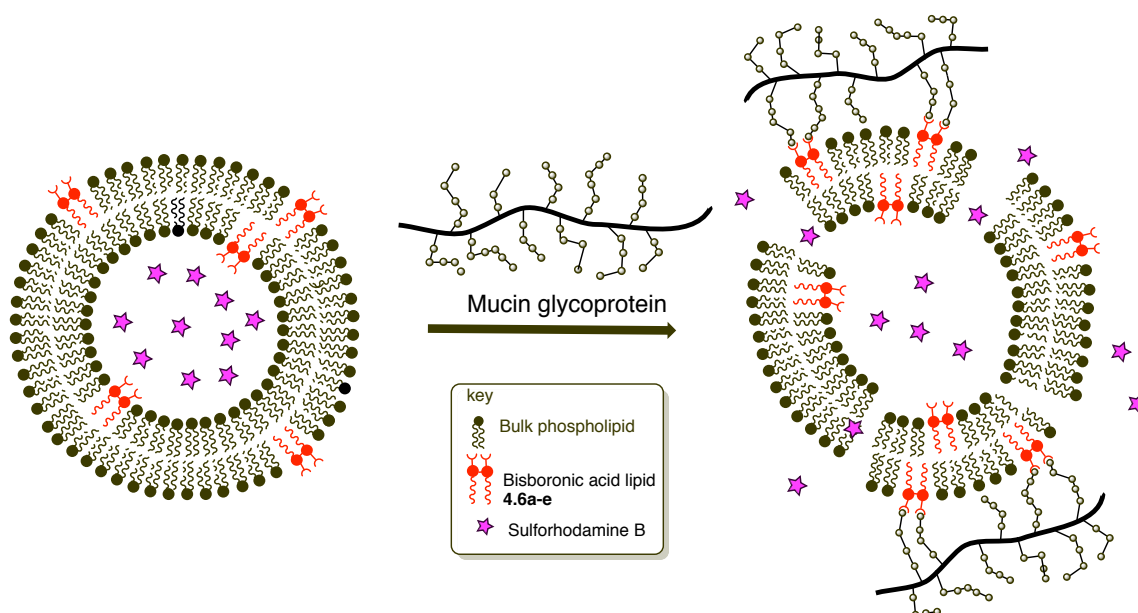


Figure 4.7. Fluorescence-based Sulforhodamine B dye leakage assays driven by mucin – bisboronic acid lipids interactions.

Hydrophilic Sulforhodamine B dyes were encapsulated within liposome aqueous interiors. Liposomes were then titrated with mucin glycoprotein and evaluated for the release of contents as judged by fluorescence increases, attributed to membrane distortion upon carbohydrate binding.

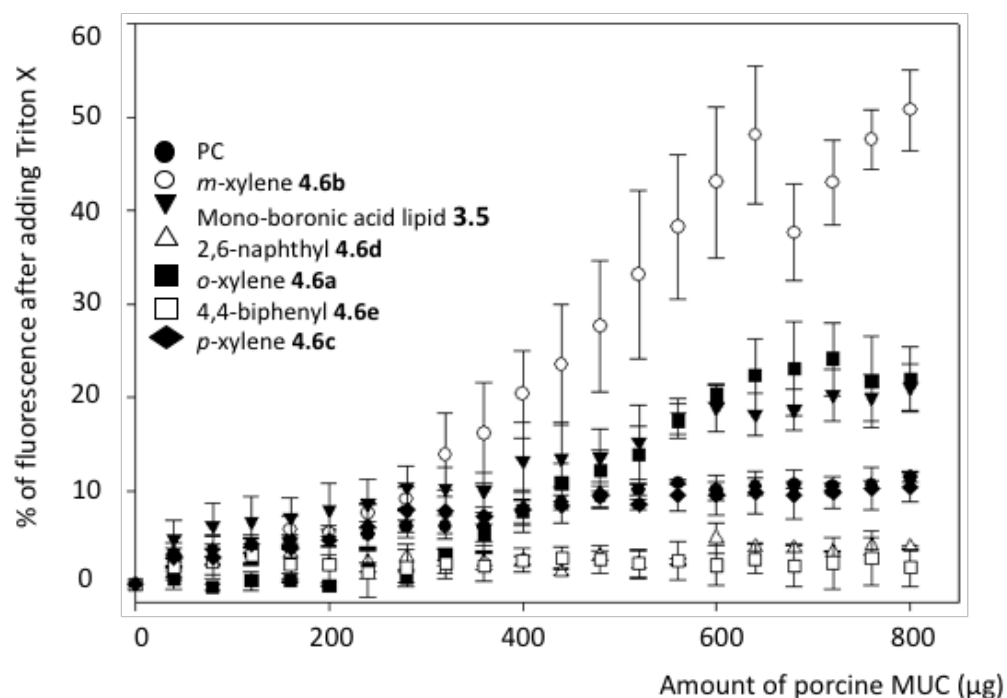


Figure 4.8. Bisboronic acid liposome Sulforhodamine B dye release results upon mucin titration.

Data plotted are standardized as a percentage of the maximum fluorescence intensity caused by Triton X treatment at the end of the experiment. Liposomes containing different bisboronic acid lipids 4.6a-e showed varied fluorescence increases upon mucin titration and the results were generally in agreement with Nile red release studies. Error bars denote the standard errors of at least three replicates.

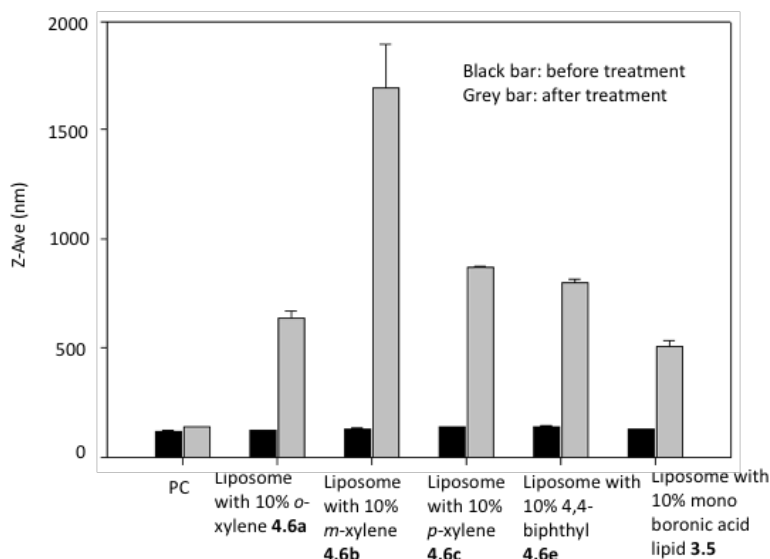


Figure 4.9. DLS results of bisboronic acid liposomes incorporating Sulforhodamine B upon mucin incubation.

DLS results showed that untreated liposomes exhibit the expected sizes based on liposome preparation through extrusion, while treatment of liposomes containing 10% bisboronic acid lipids 4.6a-e with mucin leads to larger particle sizes attributed to lipid reorganization. Among them, liposomes containing compound 4.6b had the most significant size increase, which matches the efflux data above. Error bars denote the standard errors of at least three replicates.

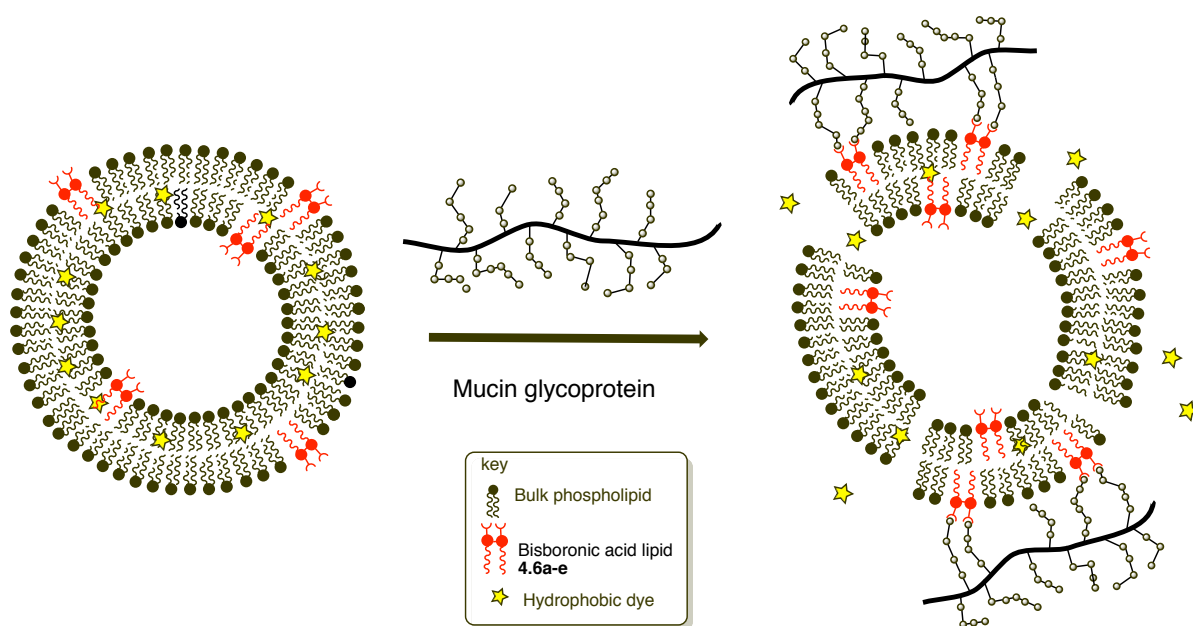


Figure 4.10. Fluorescence-based dye leakage assays driven by mucin – bisboronic acid lipid interactions.

The hydrophobic dye Nile Red was encapsulated within bisboronic acid liposome membrane bilayers. It was expected that when the bisboronic acid moieties reacted with diols in the sialic acid-rich glycoprotein mucin, liposome membranes would be disrupted and cause dye release. This leads to a decrease in Nile red fluorescence upon release since this dye is insoluble in aqueous solution.

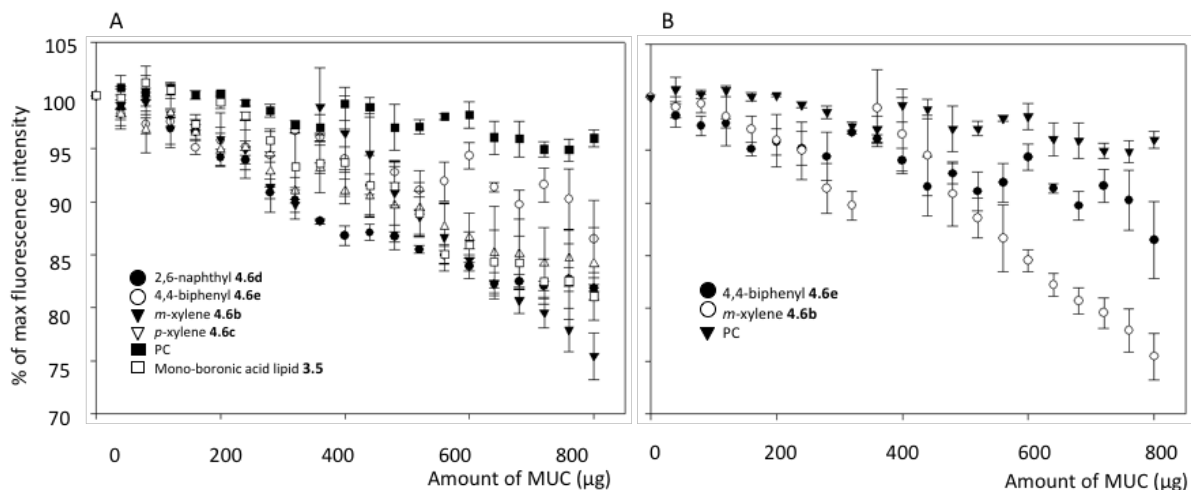


Figure 4.11. Results for release of Nile red from 5mM solutions of bisboronic acid liposomes upon mucin incubation.

(A) Decreases in Nile red fluorescence were again varied based on the particular bisboronic acid lipids of type 4.6a-e that were incorporated into liposomes. **(B)** The most significant decrease in Nile red fluorescence was from liposomes with *m*-xylene 4.6b and the least amount of decrease was from liposomes with 4,4-biphenyl 4.6e. Background release from liposomes containing only PC was minimal. Error bars denote the standard errors of at least three replicates.

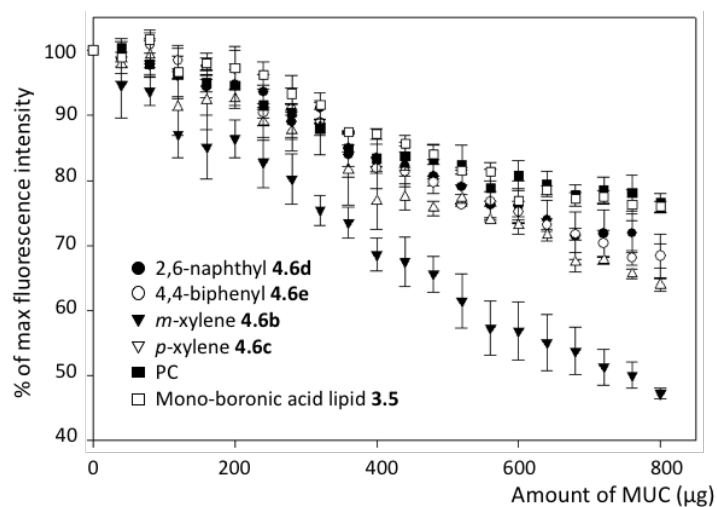


Figure 4.12. Results from Nile red release from solutions of 500 μM of bisboronic acid liposomes upon mucin incubation.

Similar trends for Nile red release have been observed when liposomes were diluted to 500 μM . However, the overall release of Nile red, including PC control liposomes lacking 4.6a-e, was more significant (ie ~20% background release from control PC liposomes) compared to results from 5mM scale studies. Error bars denote the standard errors of at least three replicates.

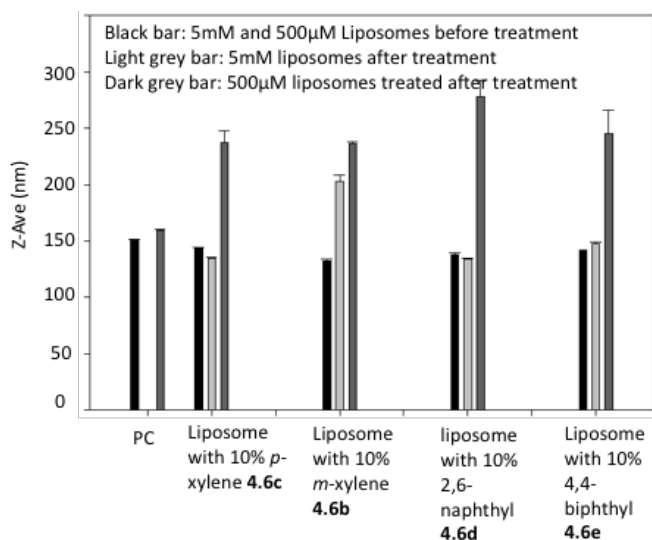


Figure 4.13. DLS results showing changes of particle sizes of liposomes before and after mucin incubation.

DLS results show that untreated liposomes exhibit the expected sizes, while treatment of liposomes containing bisboronic acid lipids 4.6b-e with mucin led to slightly larger particle sizes attributed to binding interactions and changes in lipid assembly. Studies done at the 5 µM scale showed more significant size changes compared to those at 5 mM scale. Error bars denote the standard errors of at least three replicates.

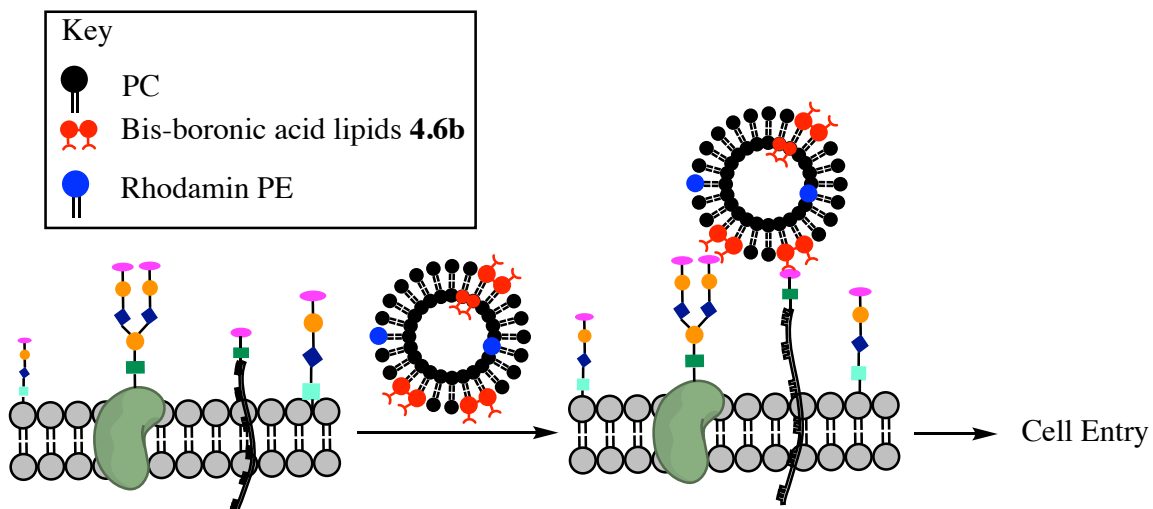


Figure 4.14. Cartoon depicting expected cellular delivery using bisboronic acid liposomes.

Cartoon description of targeted delivery of fluorescent liposomes to cells through the interaction between surface glycans and bisboronic acid lipid **4.6b**. PC-based liposomes with rhodamine-PE and/or compound **4.6b** were feed to melanoma cells to facilitate liposome cell delivery, which was assessed by fluorescence microscopy.

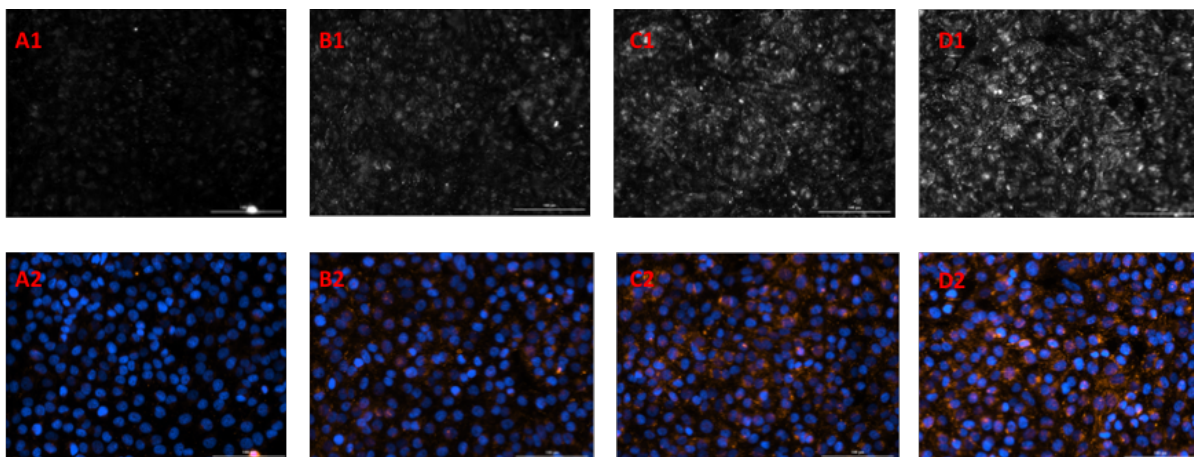
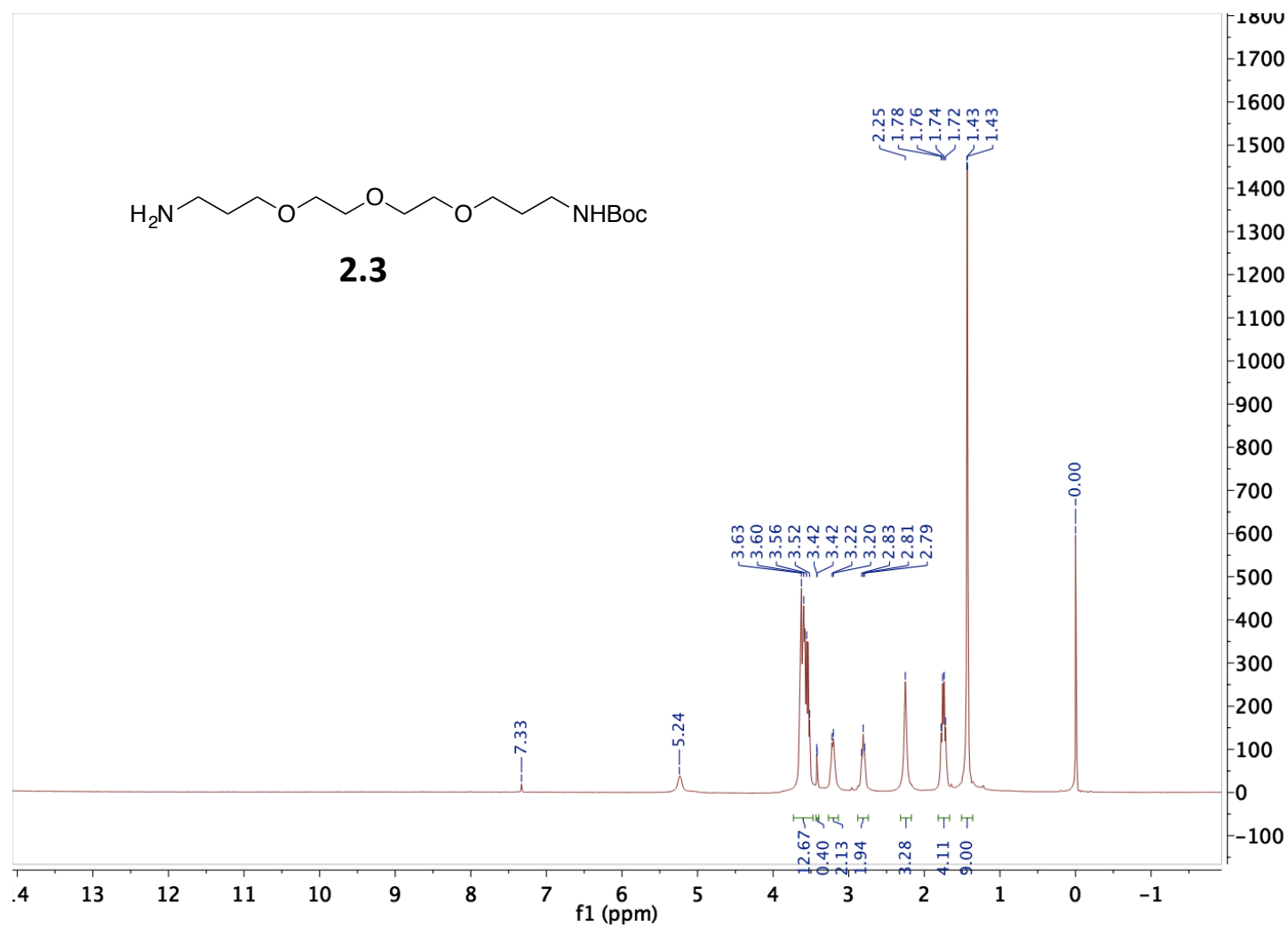


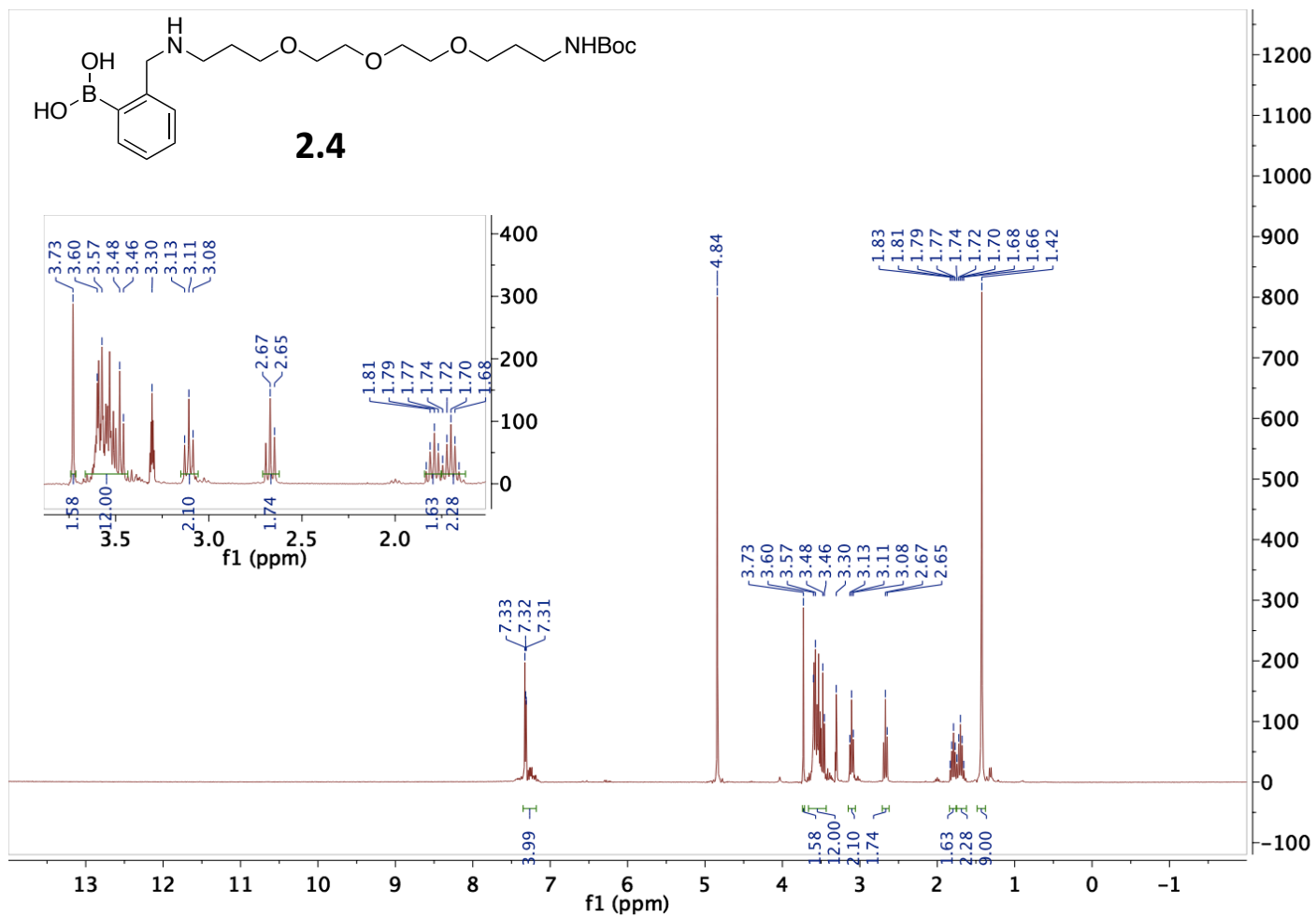
Figure 4.15. Bisboronic acid lipids 4.6b binds to melanoma cells.

Figure A1, B1, C1, D1 (black and white) and Figure A2, B2, C2, D2 (blue for DAPI and orange for Rd-PE) are images collected from confocal microscopy experiments that were merged together using the software ImageJ. A375 cells were treated for 1 hour with 1 mM of LUVs containing 0.08% rhodamine PE, PC in the absence (A1, A2) or presence of 2% (B1, B2), 5% (C1, C2), 10% (D1, D2) bisboronic acid lipid 4.6b. Figure depicts a representative image of 3 independent experiments.

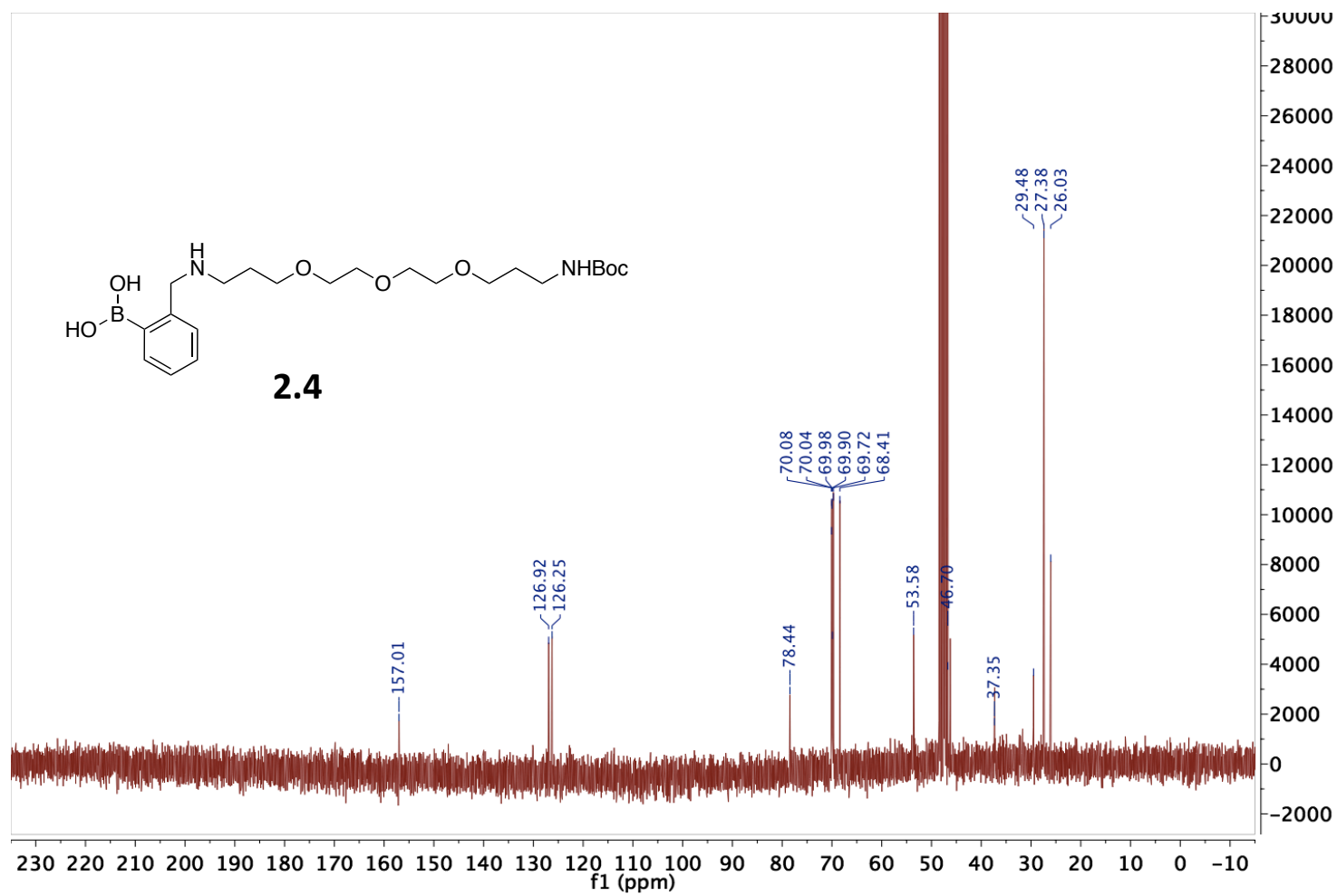
Appendix B Spectrum images



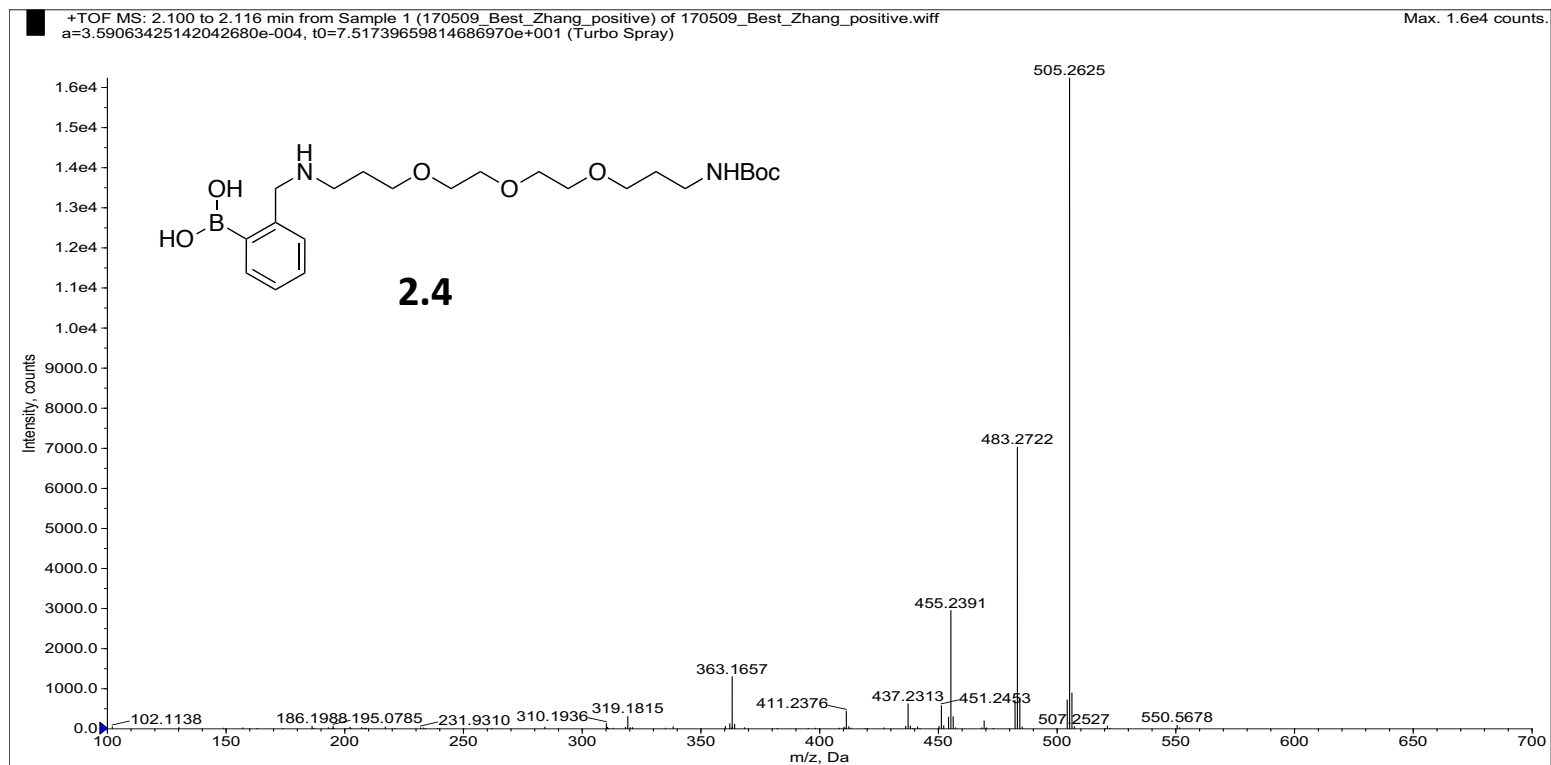
Spectrum 2.1. ¹H NMR of compound 2.3. *tert*-butyl (3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate (2.3).



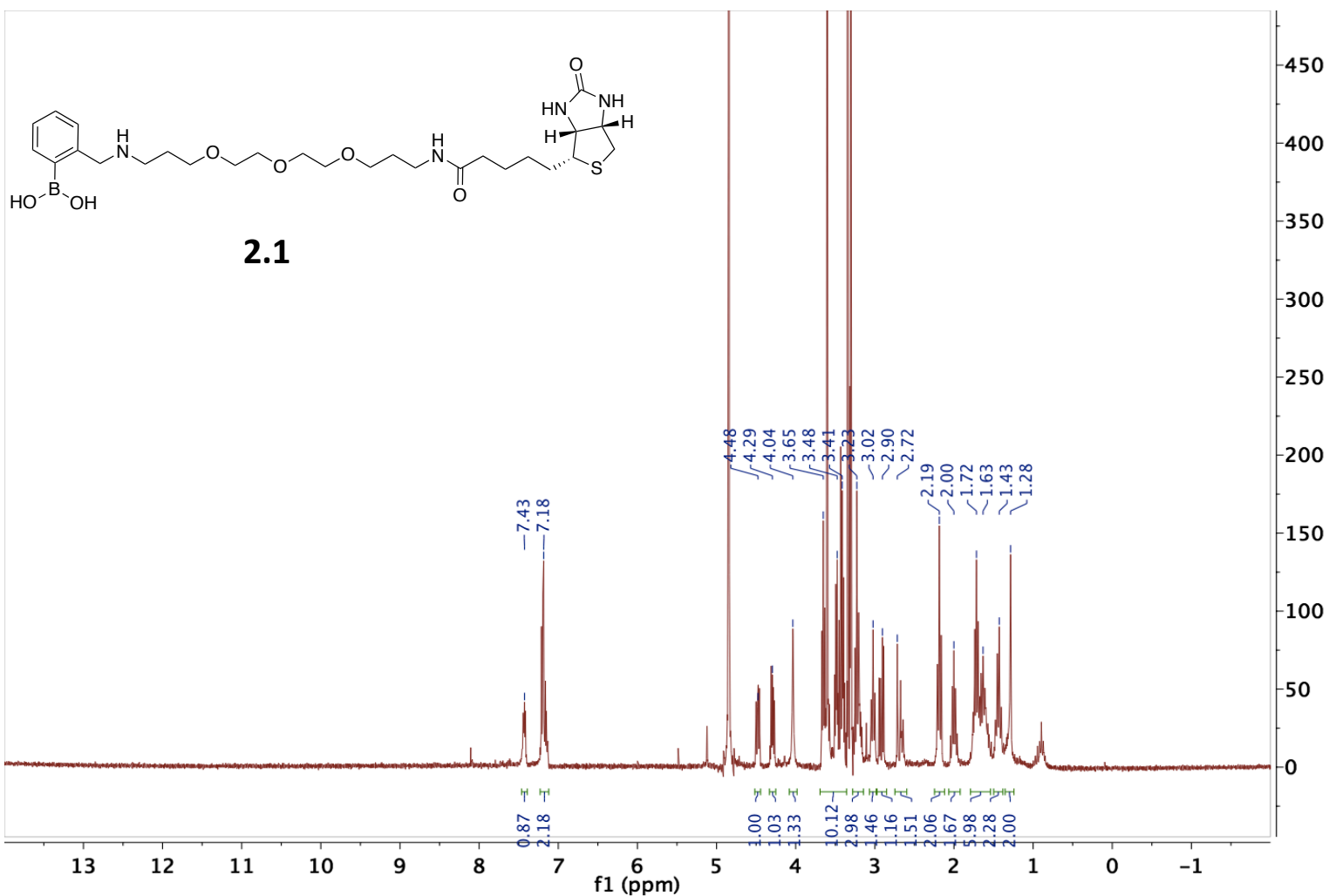
Spectrum 2.2. ^1H NMR of compound 2.4. (2-(19,19-dimethyl-17-oxo-6,9,12,18-tetraoxa-2,16-diazaicosyl)phenyl)boronic acid (2.4).



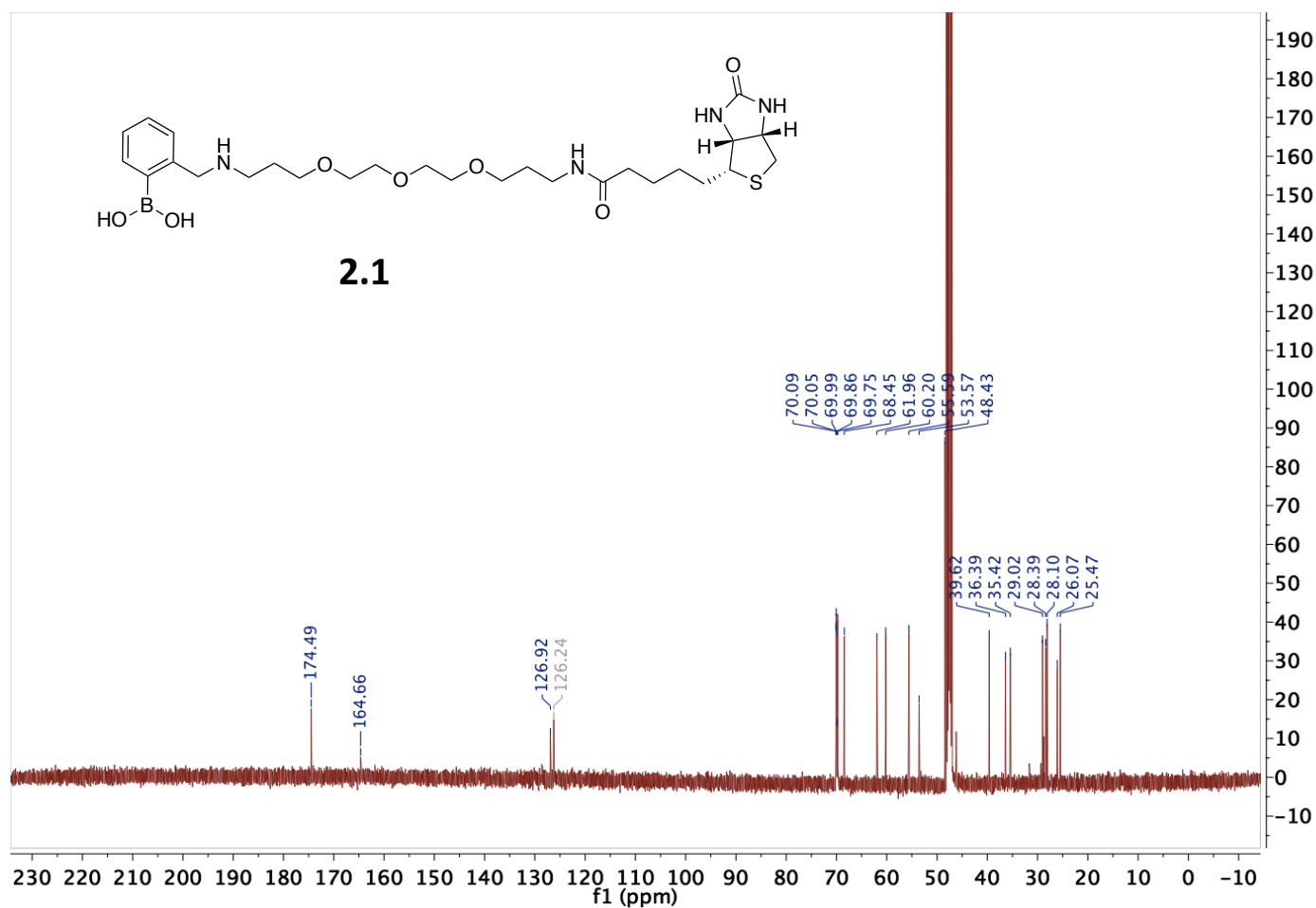
Spectrum 2.3. ¹³C NMR of compound 2.4. (2-(19,19-dimethyl-17-oxo-6,9,12,18-tetraoxa-2,16-diazaicosyl)phenyl)boronic acid (2.4).



Spectrum 2.4. Mass spectra of compound 2.4. *tert-butyl* (3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate (4.4).



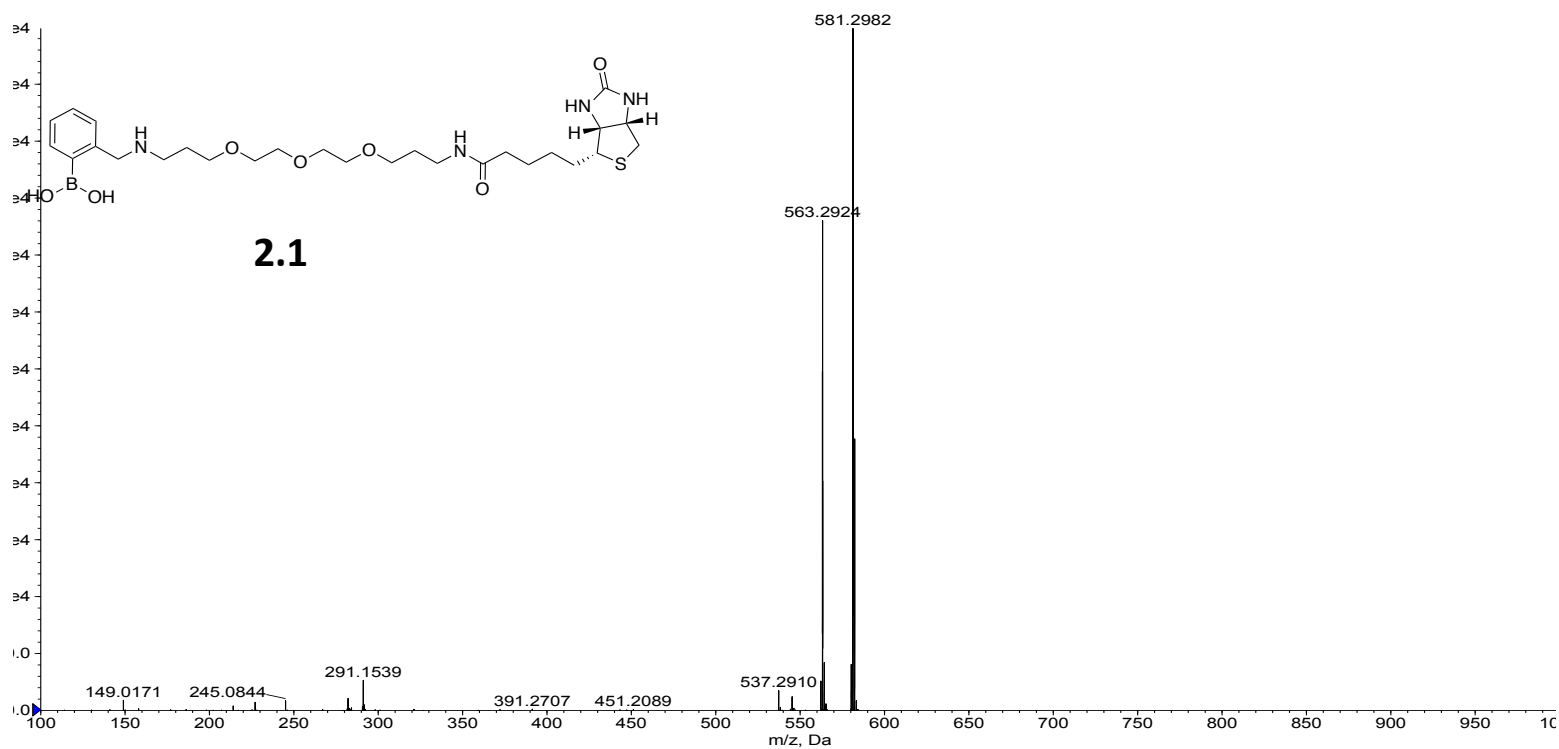
Spectrum 2.5. ^1H NMR of compound 2.1. (2-(17-oxo-21-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-6,9,12-trioxa-2,16-diazahenicosyl)phenyl)boronic acid (2.1).



Spectrum 2.6. ¹³C NMR of compound 2.1. (2-(17-oxo-21-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-6,9,12-trioxa-2,16-diazahenicosyl)phenyl)boronic acid (2.1)

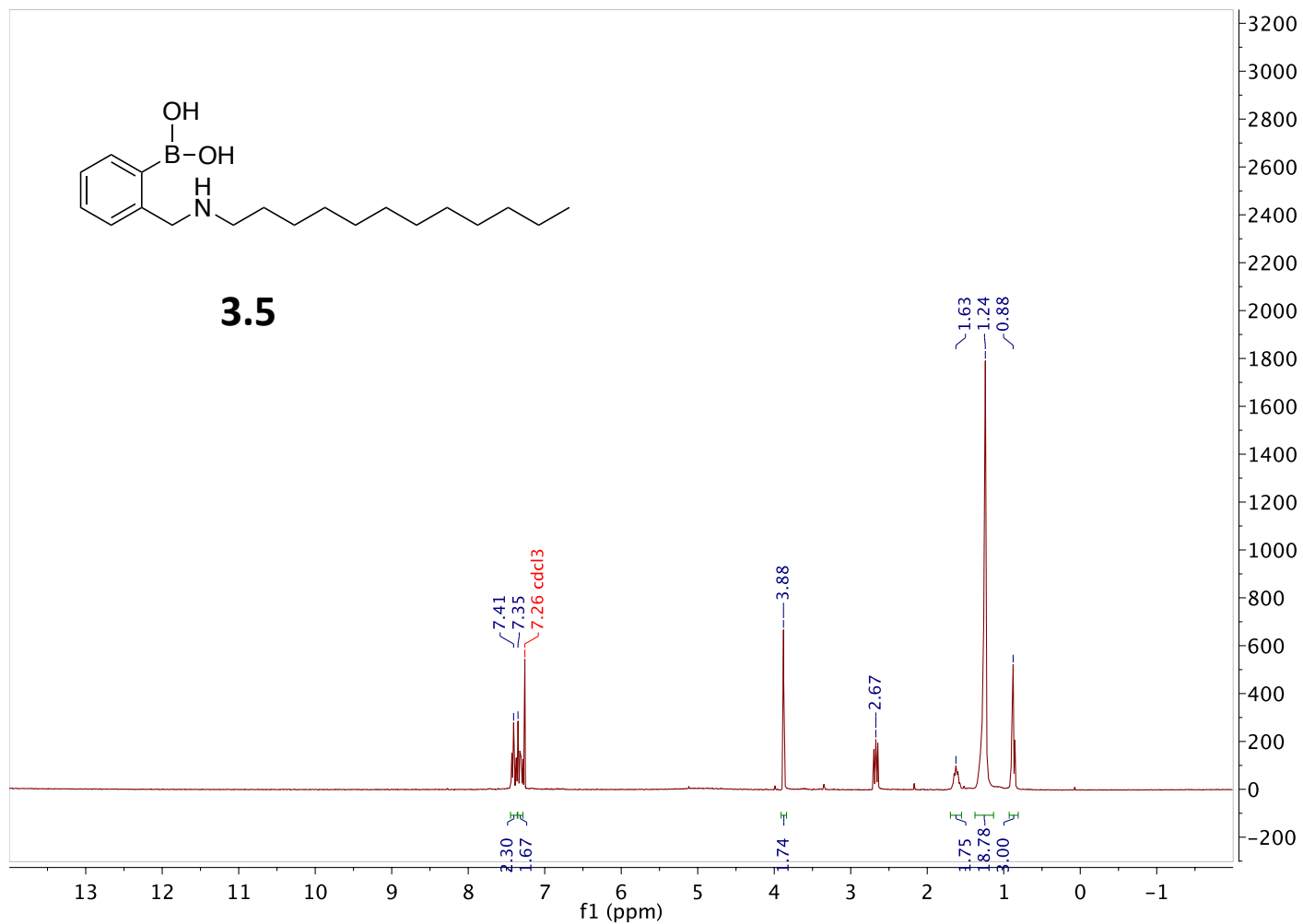
FOF MS: 1.098 to 1.115 min from Sample 1 (20160411_Best_Zhang_02) of 20160411_Best_Zhang_02.wiff
3.59081171941098590e-004, t0=7.40646874774663640e+001 (Turbo Spray)

Max. 6.0e4 cou

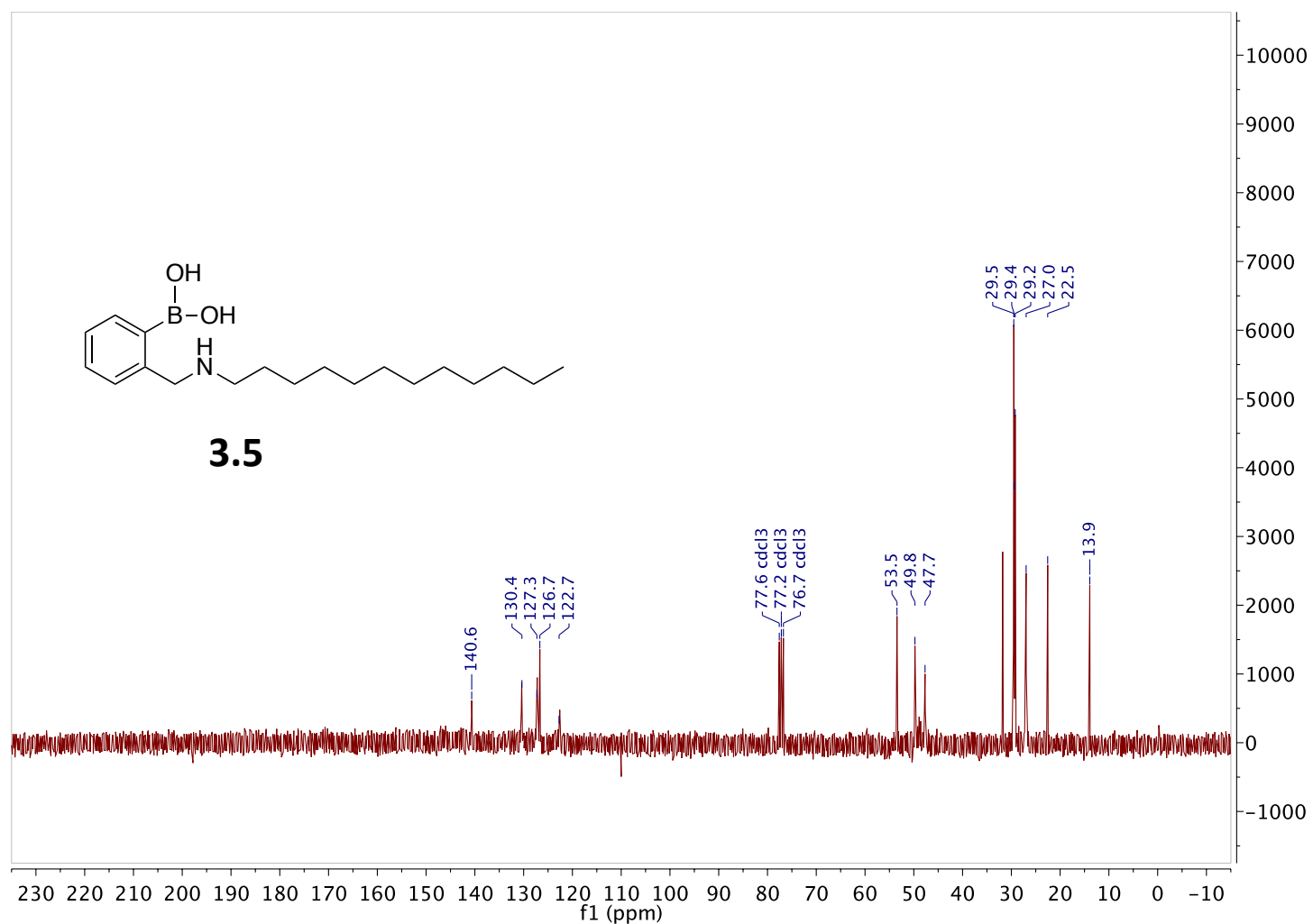


17

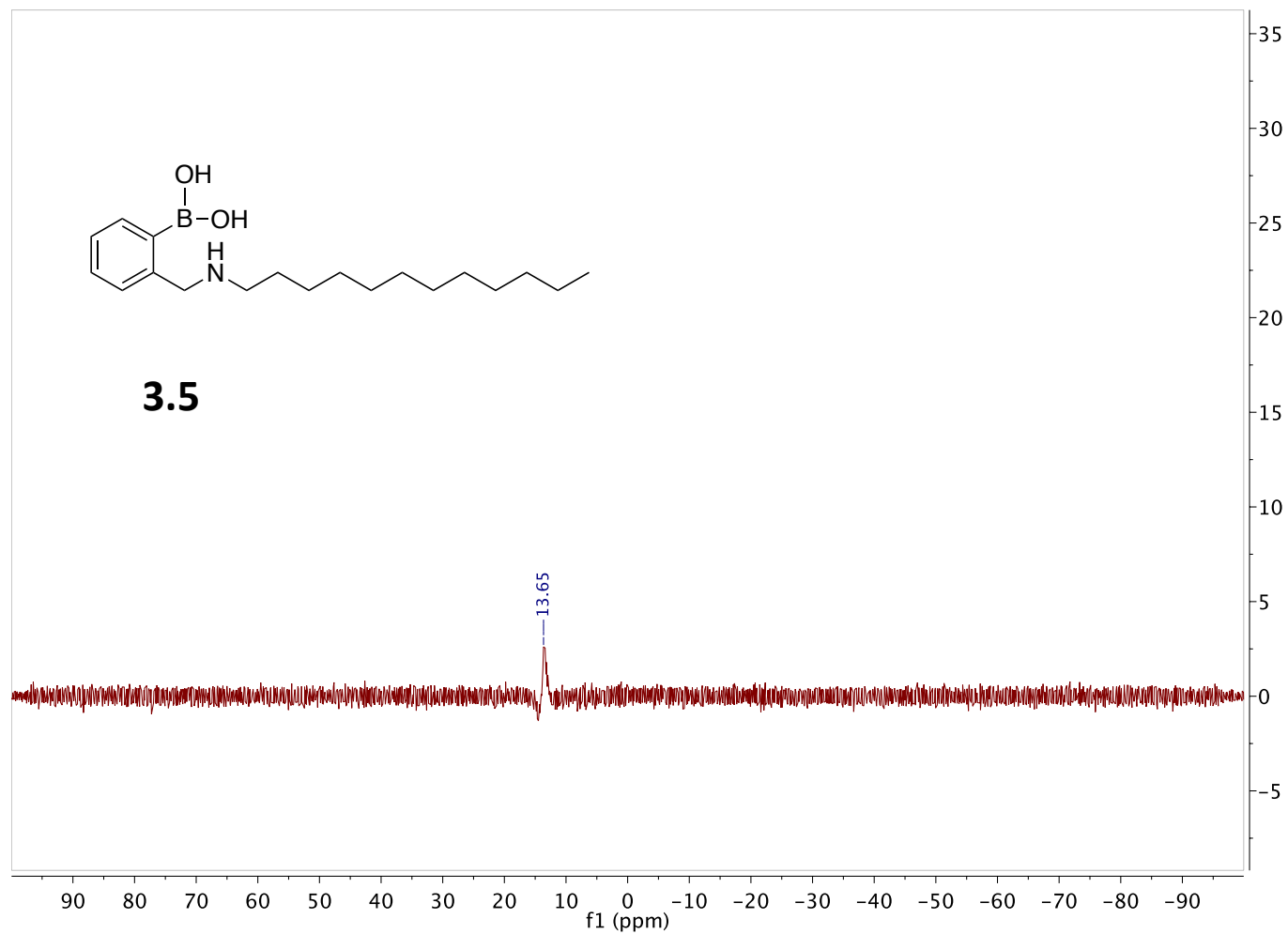
Spectrum 2.7. Mass spectra of compound 2.1. (2-(17-oxo-21-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-6,9,12-trioxa-2,16-diazahenicosyl)phenyl)boronic acid (2.1)



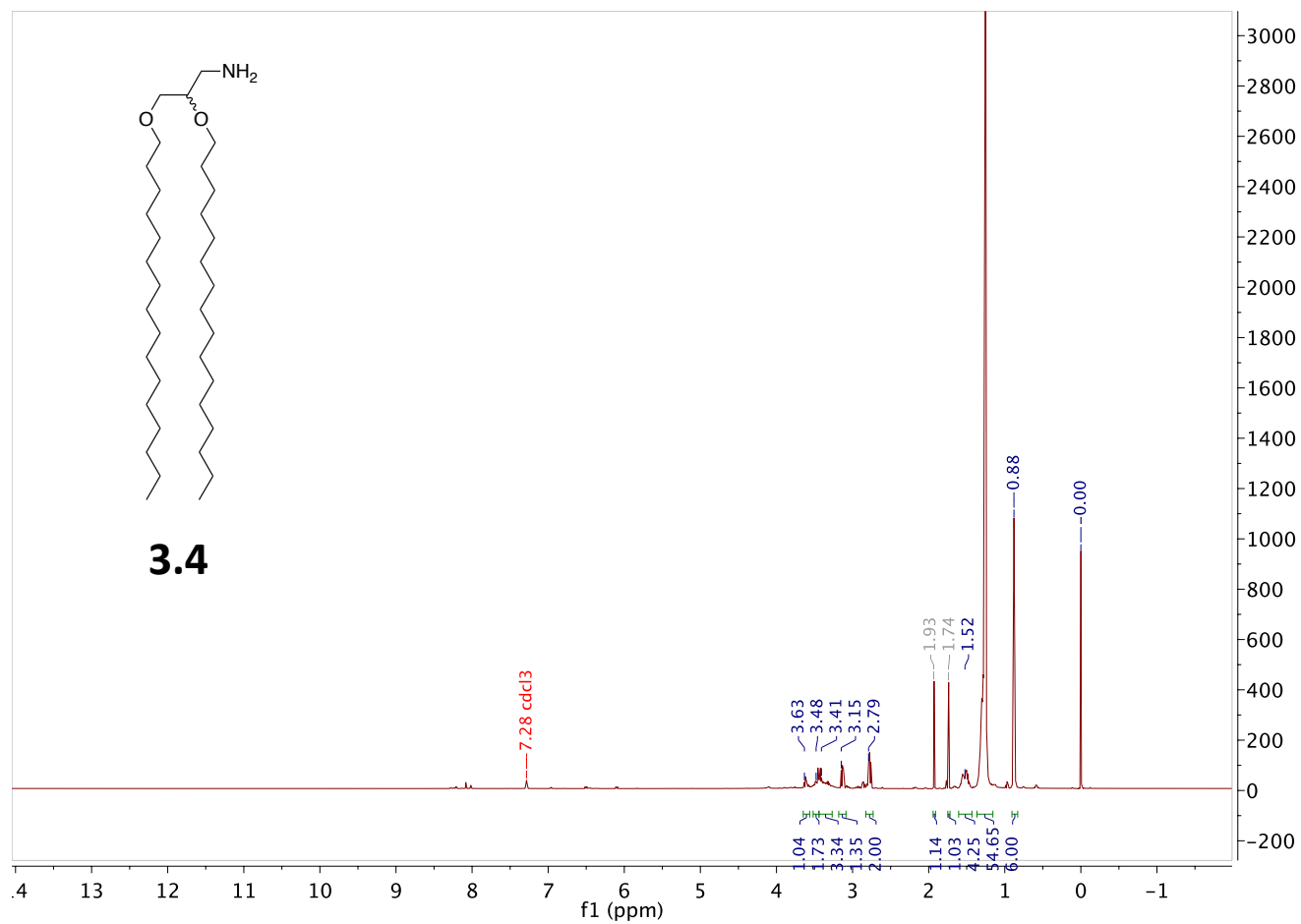
Spectrum 3.1. ¹H NMR of compound 3.5. (2-((dodecylamino)methyl)phenyl)boronic acid (3.5)



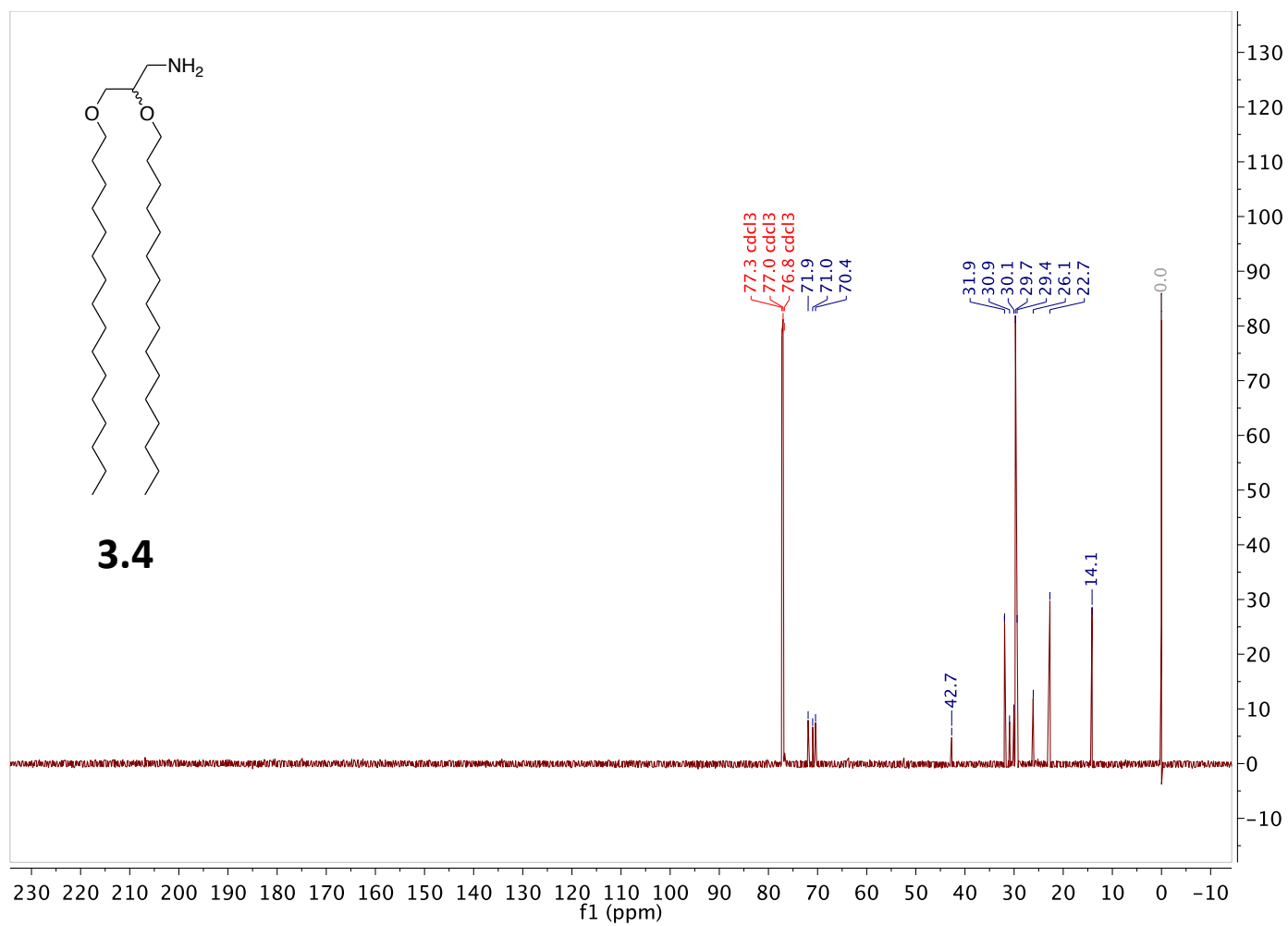
Spectrum 3.2. ¹³C NMR of compound 3.5. (2-((dodecylamino)methyl)phenyl)boronic acid (3.5)



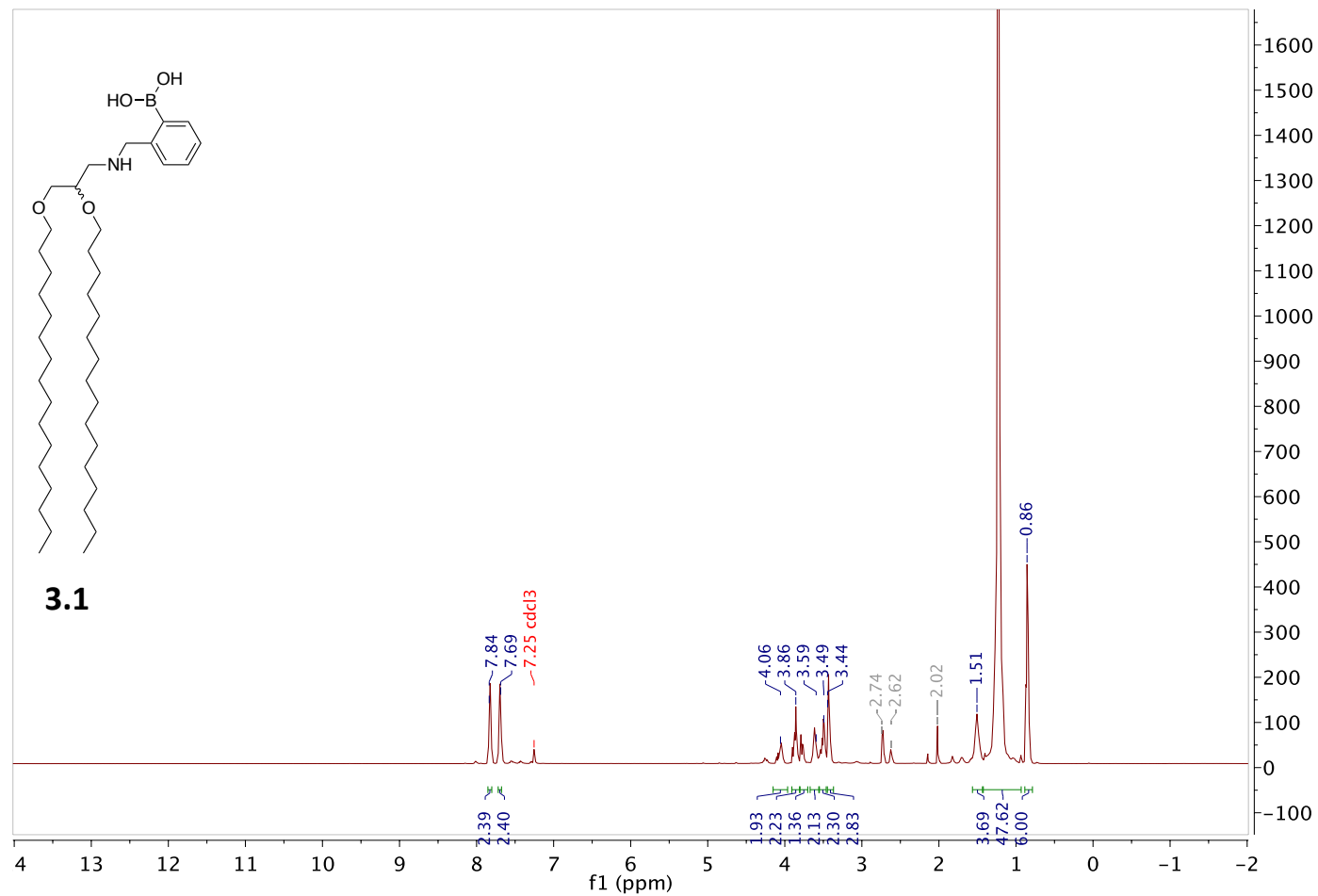
Spectrum 3.3. ¹¹B NMR of compound 3.5. (2-((dodecylamino)methyl)phenyl)boronic acid (3.5)



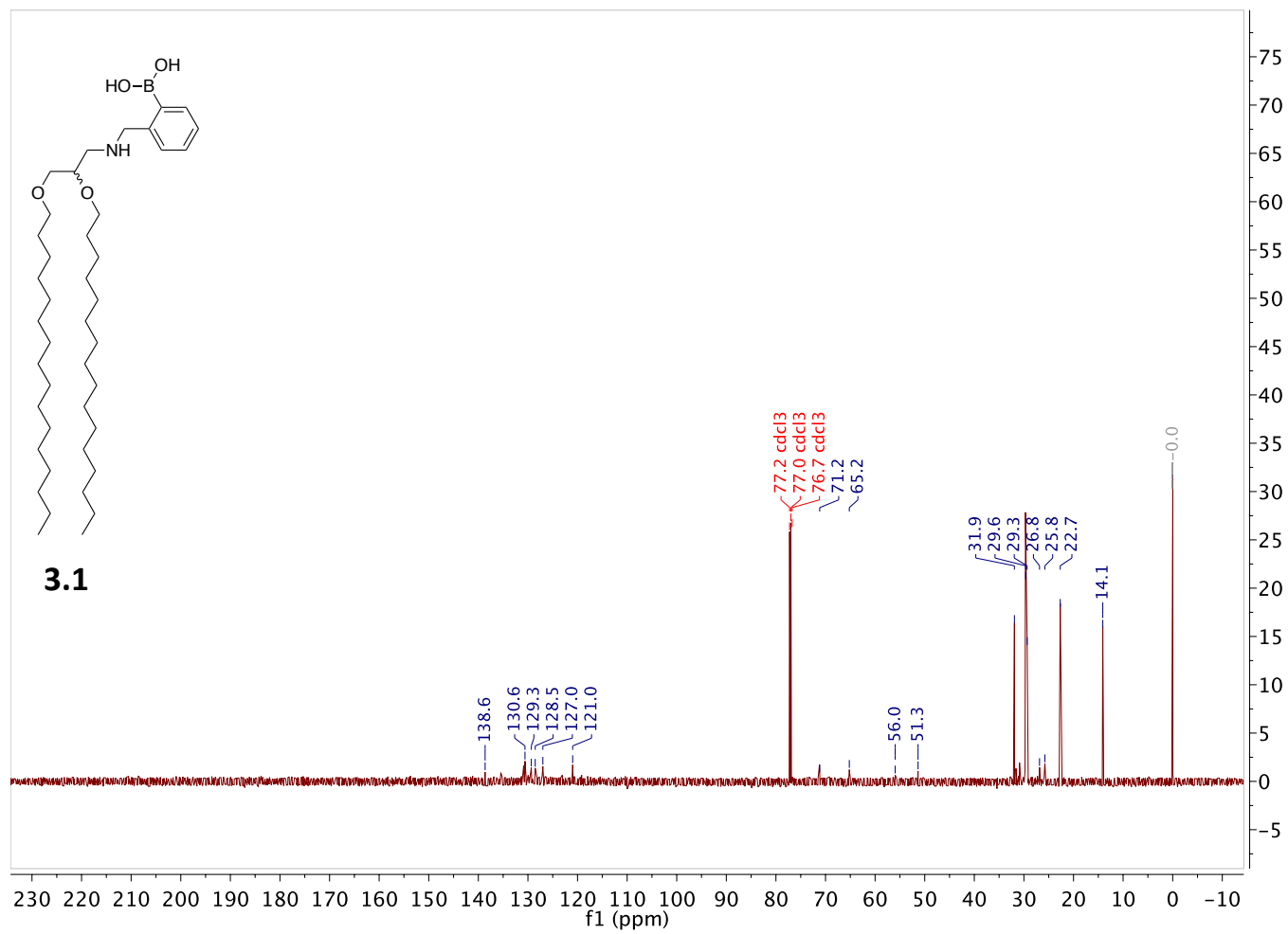
Spectrum 3.4. ¹H NMR of compound 3.5. 2,3-bis(hexadecyloxy)propan-1-amine (3.4)



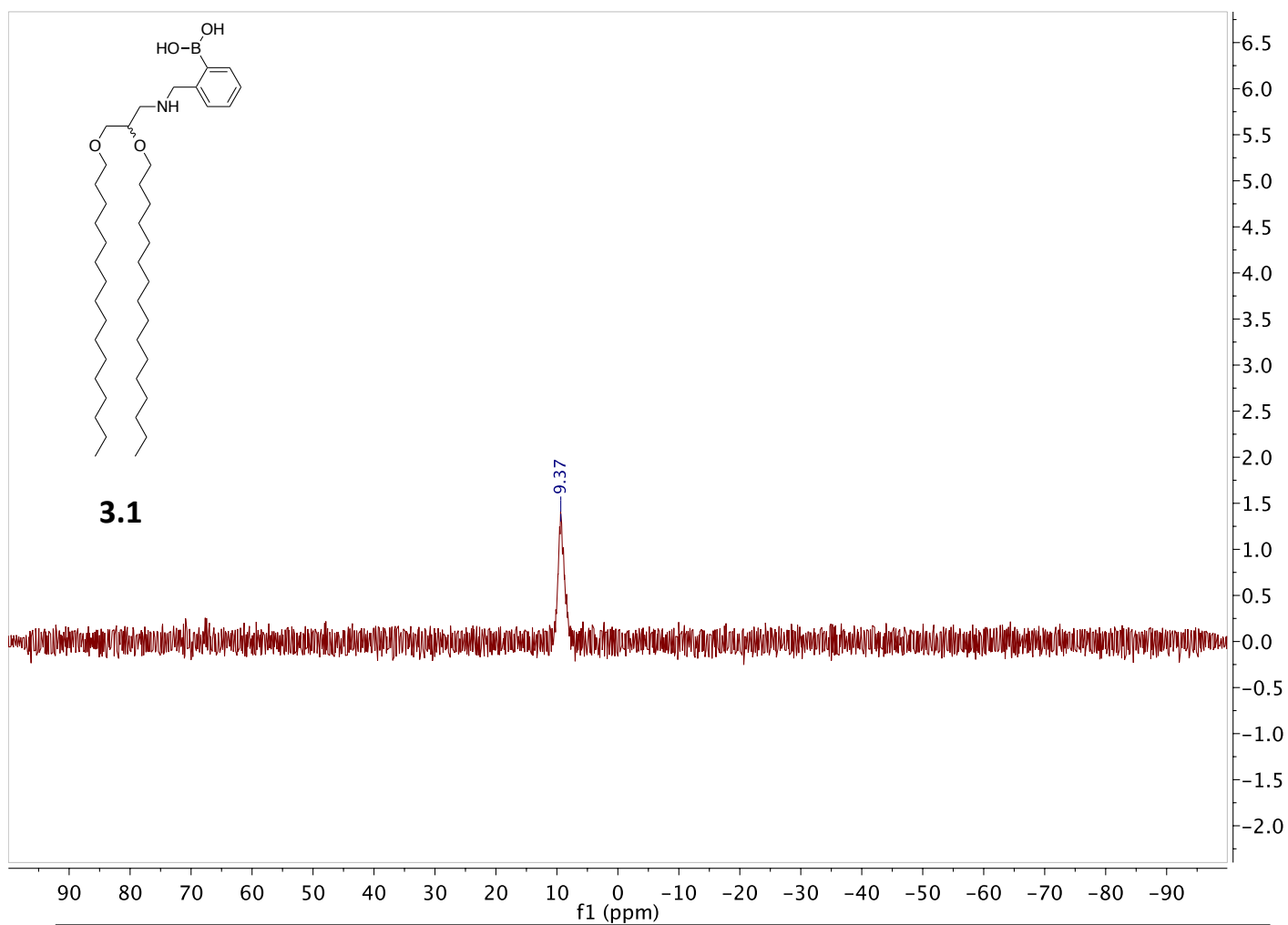
Spectrum 3.5. ^{13}C NMR of compound 3.5. 2,3-bis(hexadecyloxy)propan-1-amine (3.4)



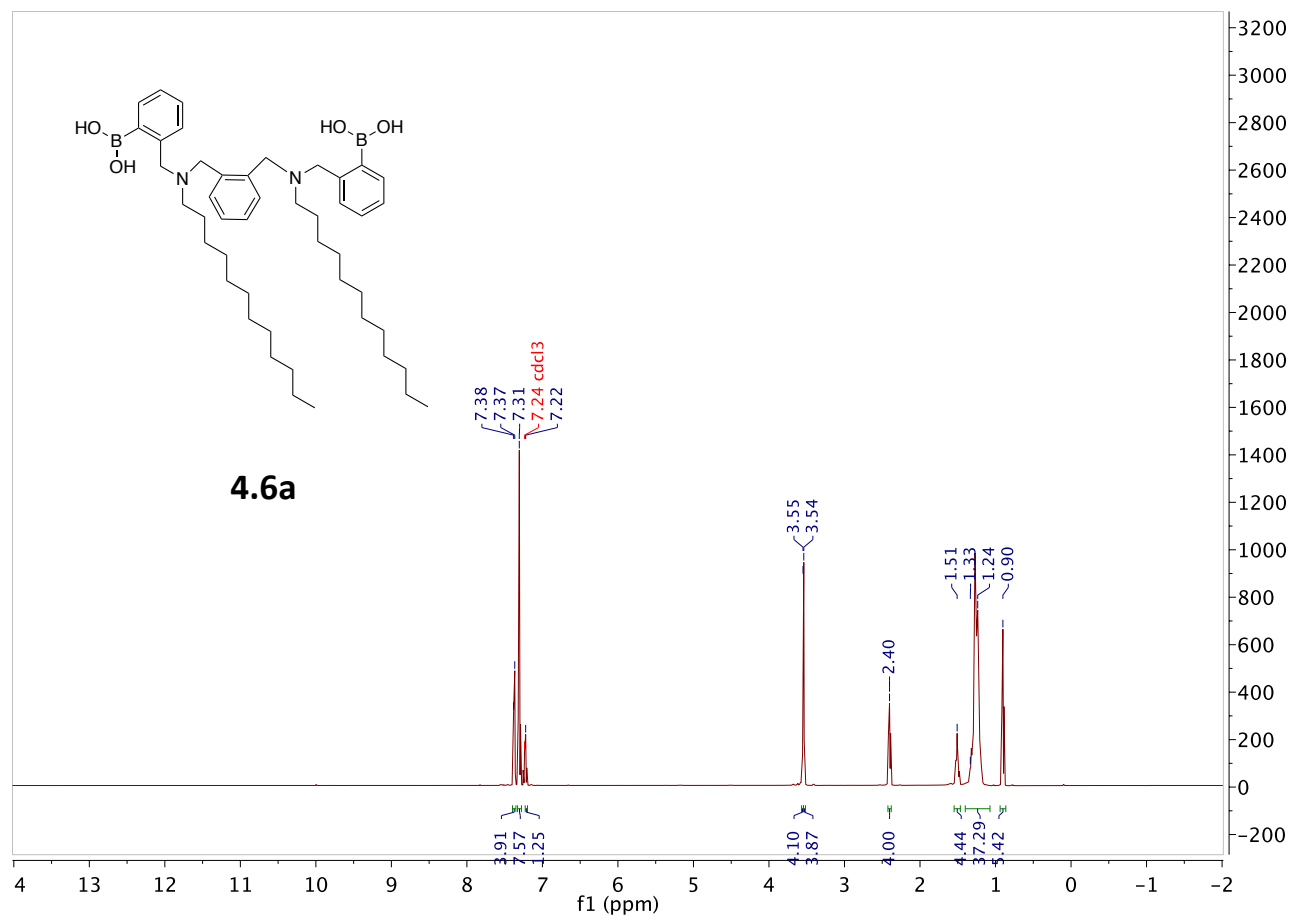
Spectrum 3.6. ¹H NMR of compound 3.1. (2-(((2,3-bis(hexadecyloxy)propyl)amino)methyl)phenyl)boronic acid (3.1)



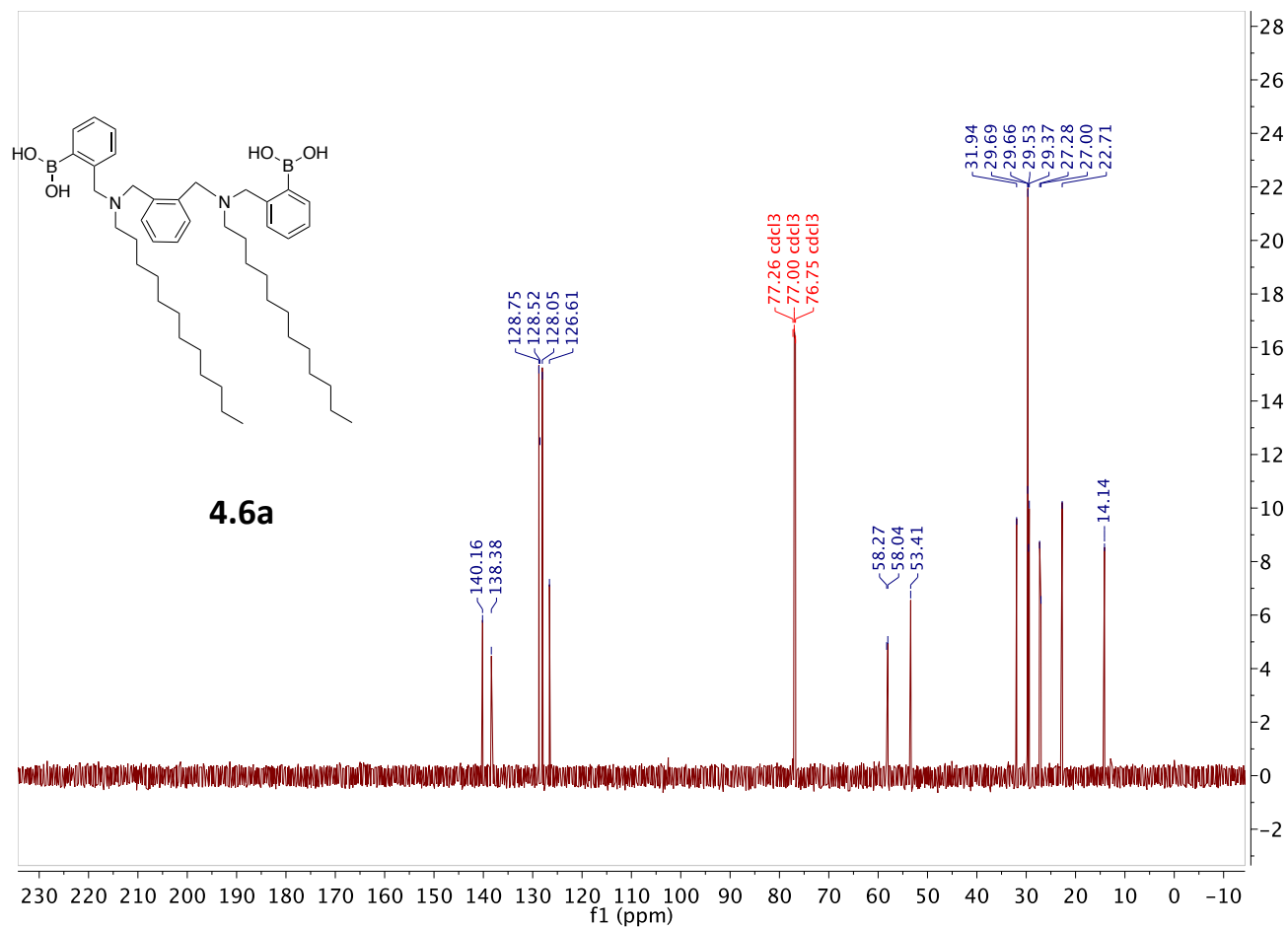
Spectrum 3.7. ^{13}C NMR of compound 3.1. (2-(((2,3-bis(hexadecyloxy)propyl)amino)methyl)phenyl)boronic acid (3.1)



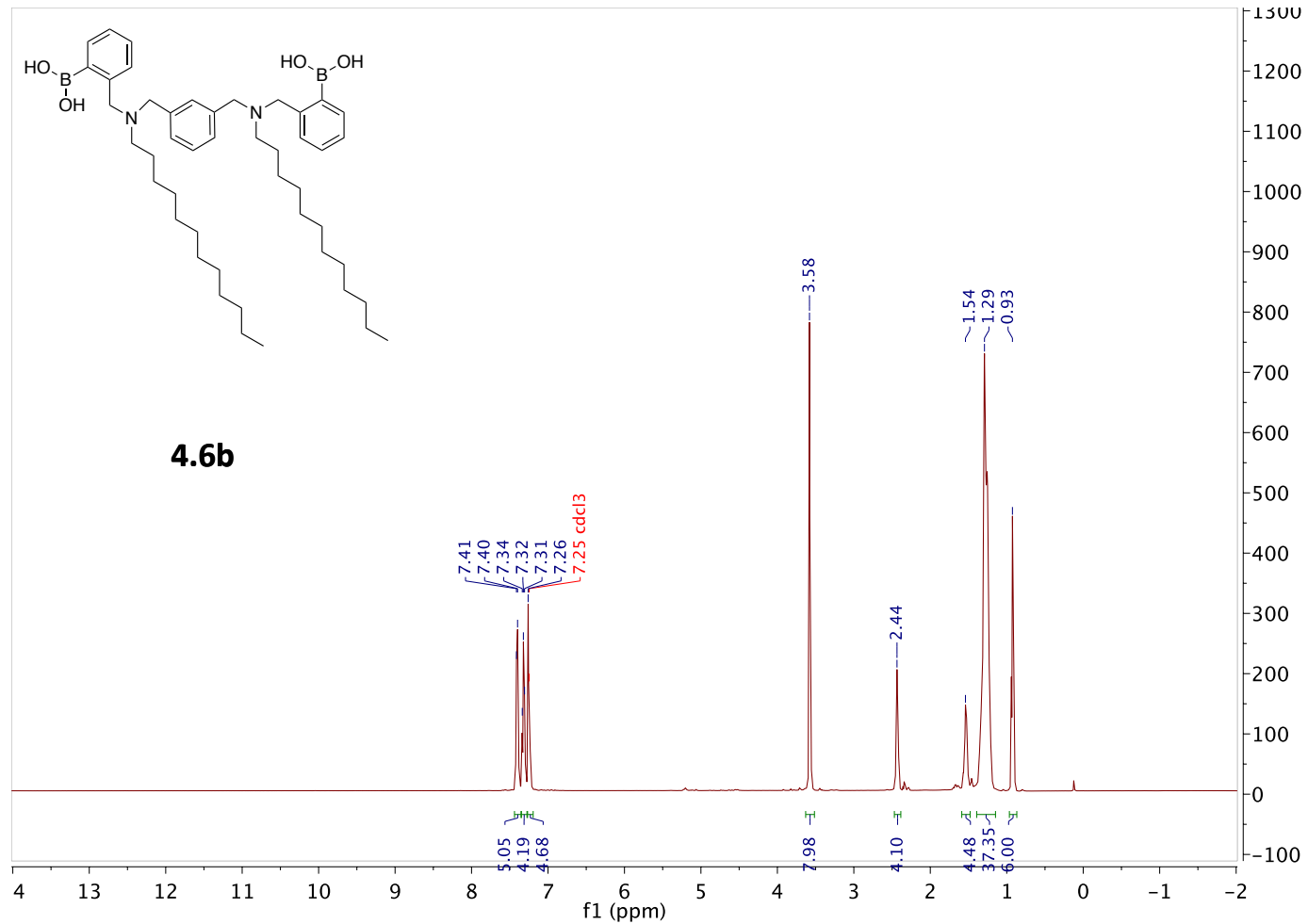
Spectrum 3.8. ^{11}B NMR of compound 3.1. (2-(((2,3-bis(hexadecyloxy)propyl)amino)methyl)phenyl)boronic acid (3.1)



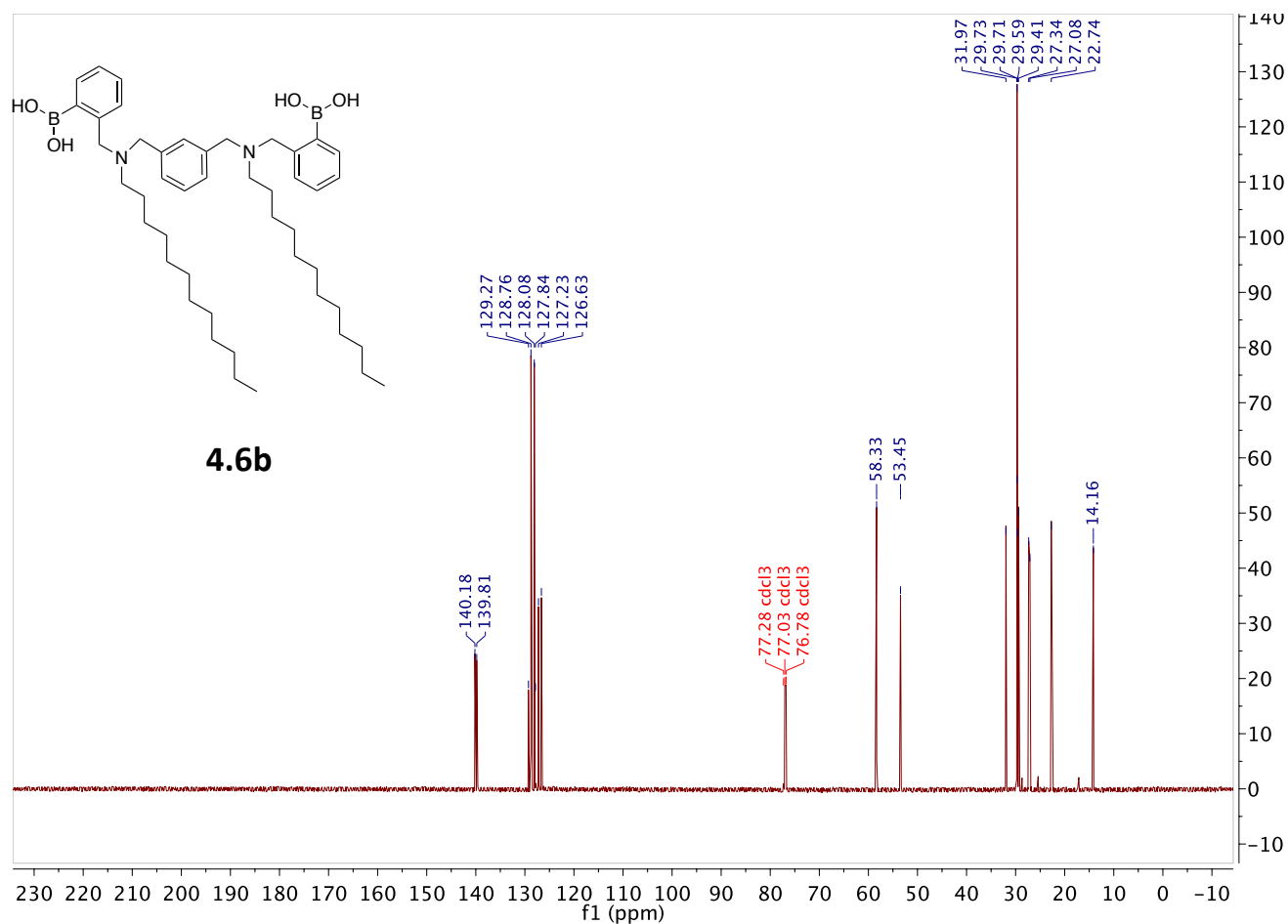
Spectrum 4.1. ¹H NMR of compound 4.6a. (((1,2-phenylenebis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene))diboronic acid (4.6a)



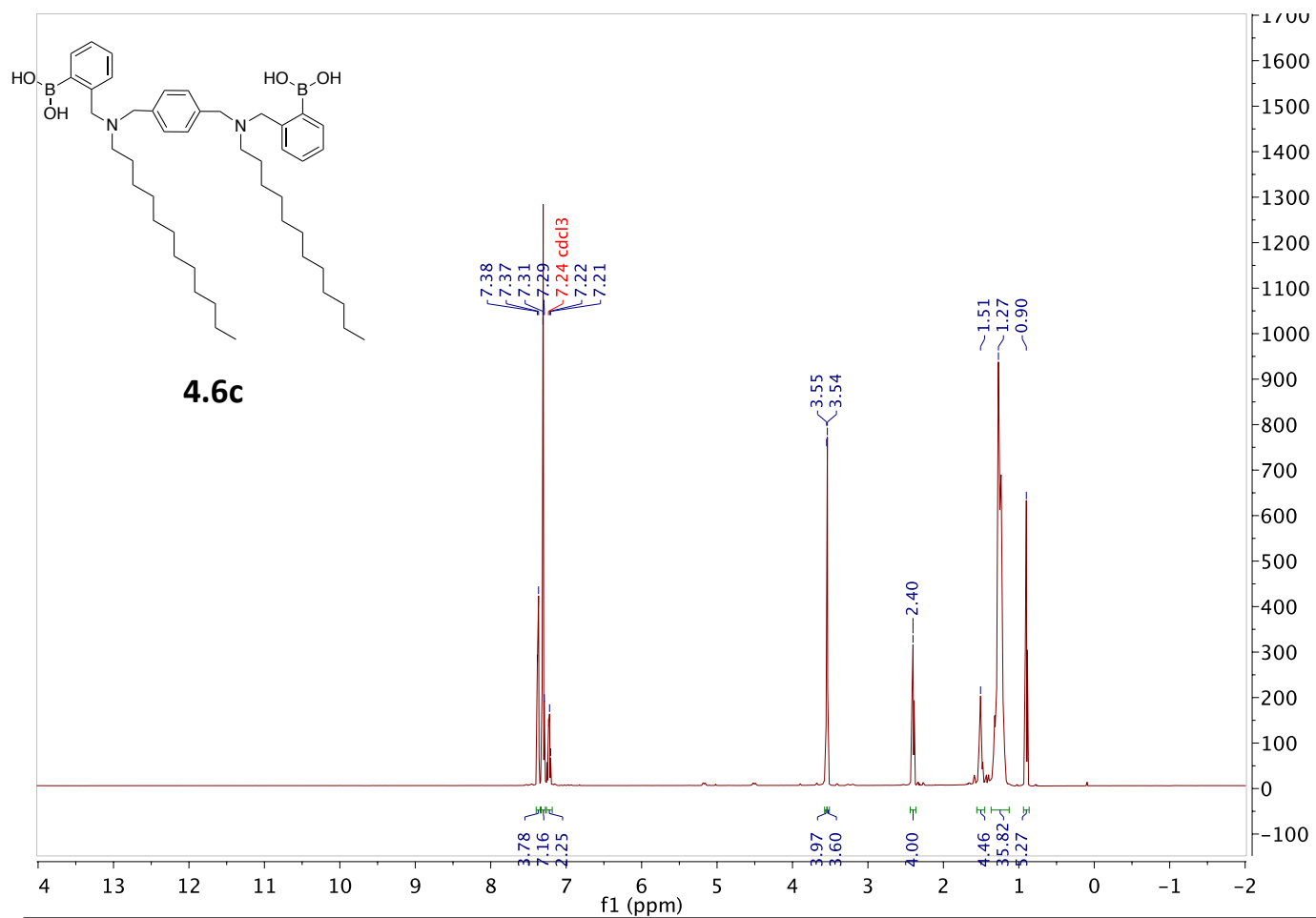
Spectrum 4.2. ¹³C NMR of compound 4.6a. (((1,2-phenylenebis(methylene))bis(dodecylazanediy))bis(methylene))bis(2,1-phenylene))diboronic acid (4.6a)



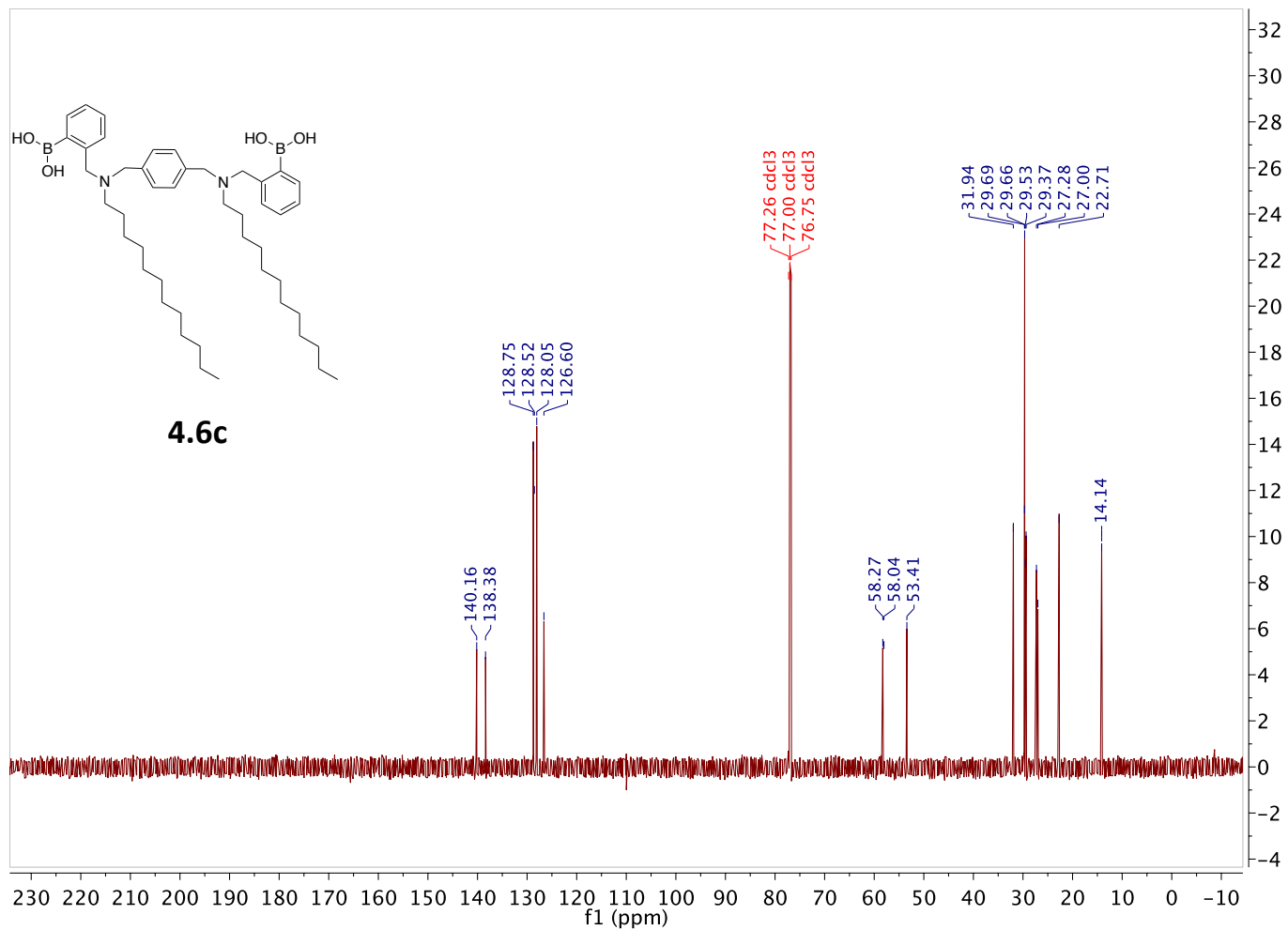
Spectrum 4.3. ¹H NMR of compound 4.6b. (((1,3-phenylenebis(methylene))bis(dodecylazanediy))bis(methylene))bis(2,1-phenylene)diboronic acid (4.6b)



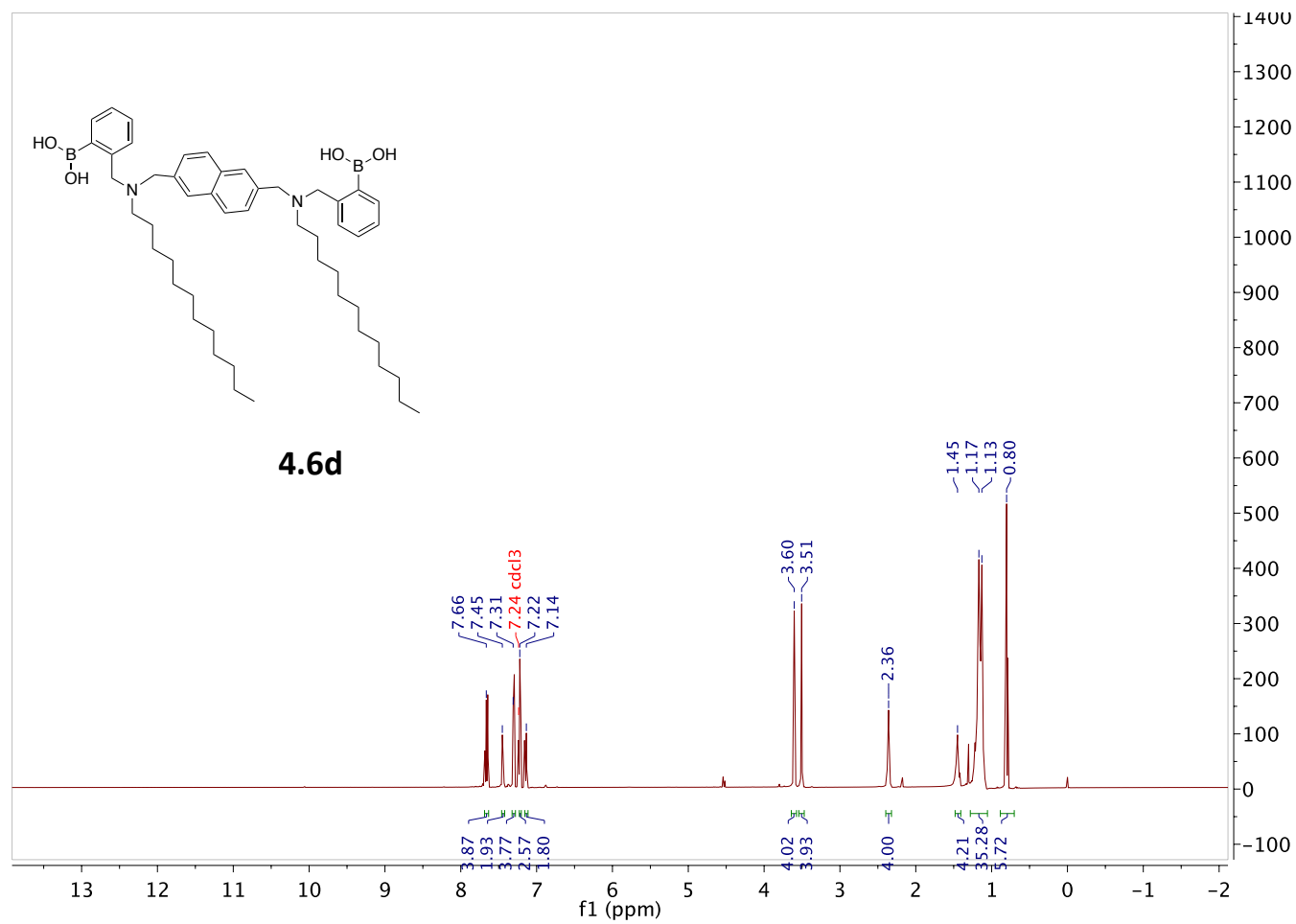
Spectrum 4.4. ^{13}C NMR of compound 4.6b. (((1,3-phenylenebis(methylene))bis(dodecylazanediy))bis(methylene))bis(2,1-phenylene))diboronic acid (4.6b)



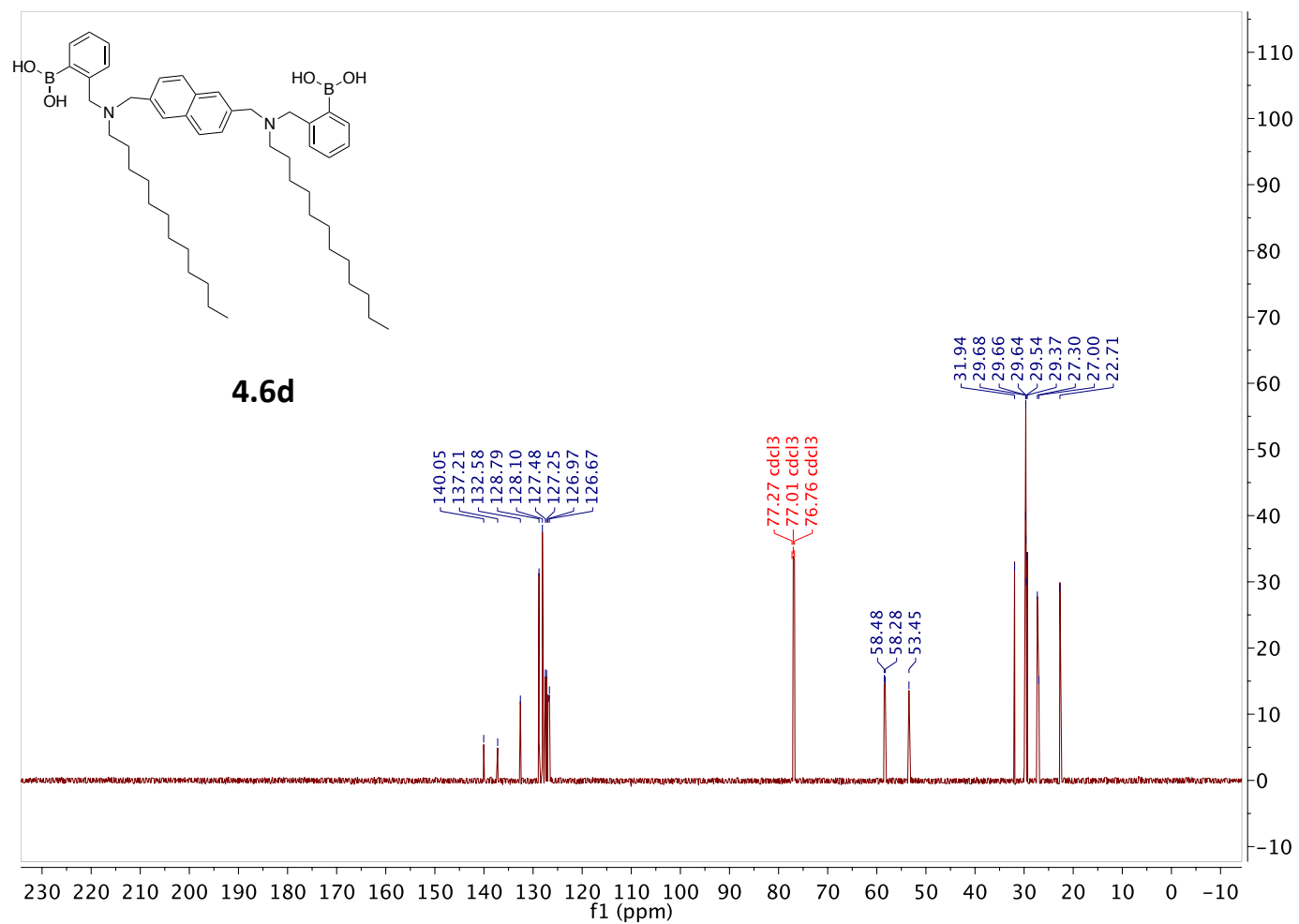
Spectrum 4.5. ¹H NMR of compound 4.6c. (((1,4-phenylenebis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene))diboronic acid (4.6c)



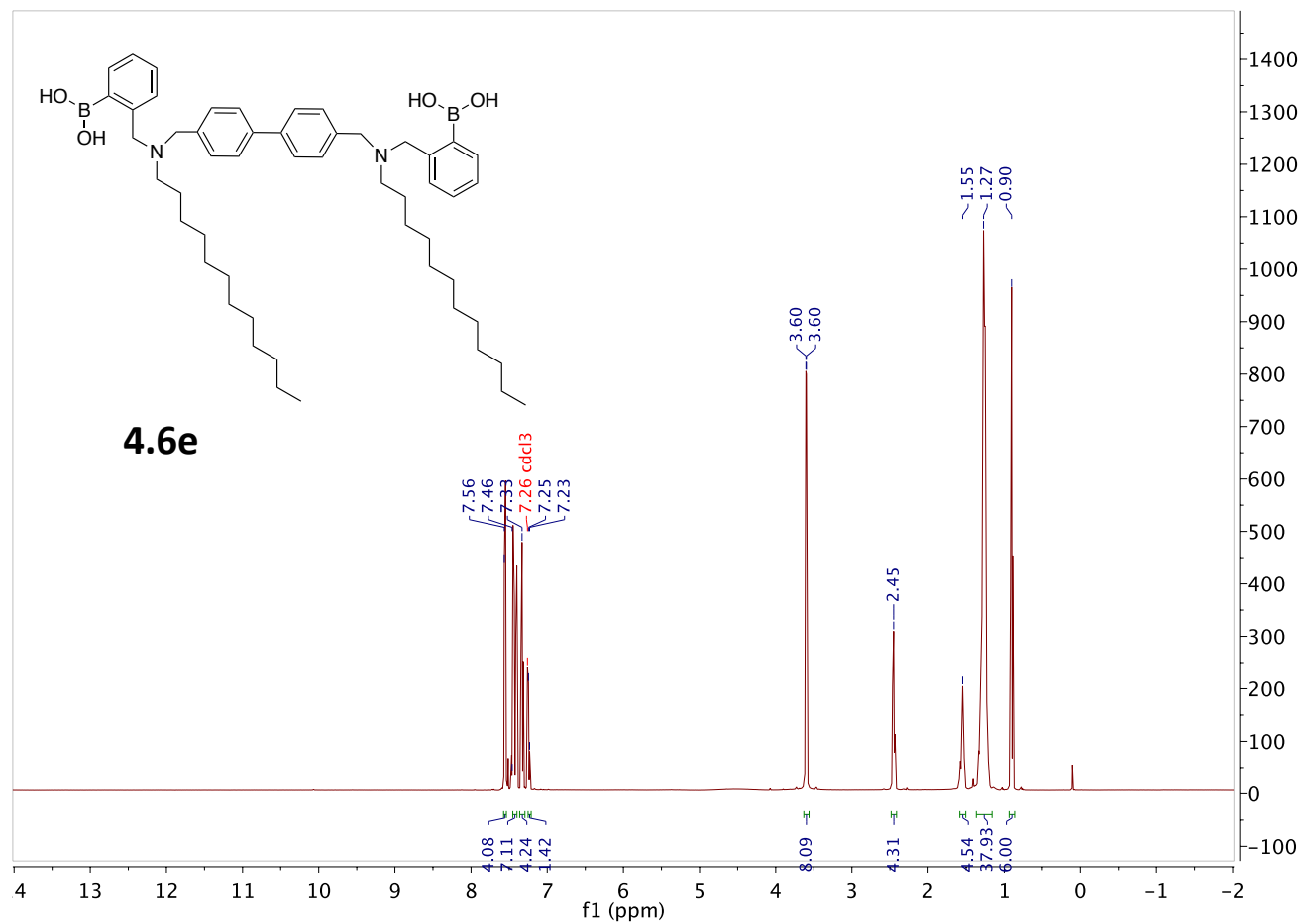
Spectrum 4.6. ¹³C NMR of compound 4.6c. (((1,4-phenylenebis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene))diboronic acid (4.6c)



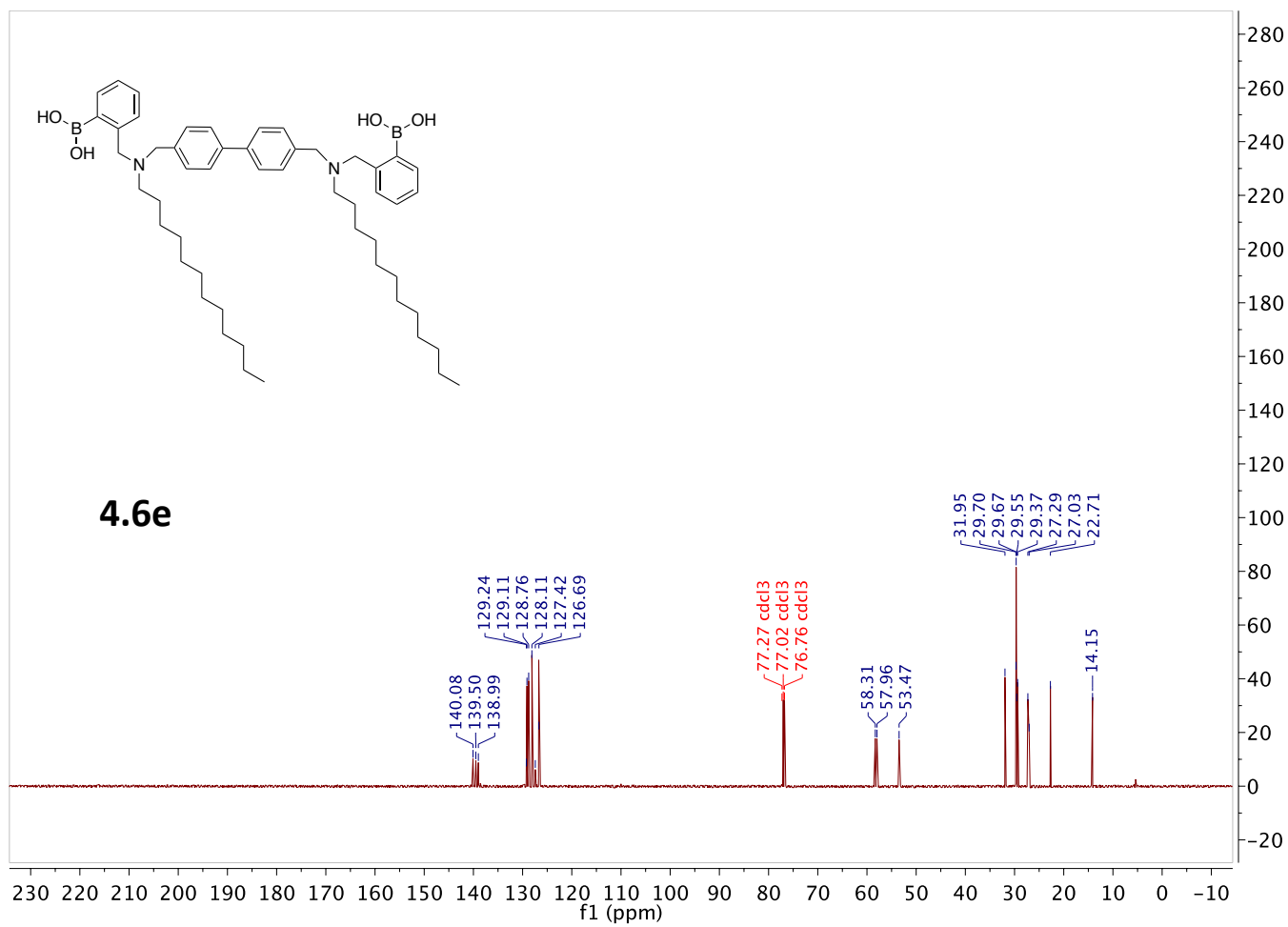
Spectrum 4.7. ¹H NMR of compound 4.6d. (((naphthalene-2,6-diylbis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene)diboronic acid (4.6d)



Spectrum 4.8. ¹³C NMR of compound 4.6d. (((naphthalene-2,6-diylbis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene)diboronic acid (4.6d)



Spectrum 4.9. ^1H NMR of compound 4.6e. ((([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(dodecylazanediy))bis(methylene))bis(2,1-phenylene))diboronic acid (4.6e)



Spectrum 4.10. ¹³C NMR of compound 4.6e. ((([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(dodecylazanediyl))bis(methylene))bis(2,1-phenylene))diboronic acid (4.6e)

Vita

Xiaoyu Zhang was born in Chengdu, China in 1992. In 2010, She began attending Shanghai Jiaotong University majoring in medical laboratory diagnosis. During her undergraduate studies, she realized her passion for exploring fundamental biology and chemistry. She then joined the chemistry department at the University of Tennessee and spent five years studying chemical biology. She served as a teaching assistant for both general and organic chemistry while doing research work in the lab of Dr. Michael Best. Her research focused on the development of boronic acid materials for cellular surface glycan recognition. Just right before graduation, she married Dr. Shutang (Steve) You, who is an electrical engineer working in the EECS Department at UT. In her spare time, Xiaoyu likes reading novels, cooking, barre and hiking around beautiful east Tennessee with family and friends.