THE SYNTHESIS AND CHARACTERIZATION OF DENDRONIZED BIOMACROMOLECULES

THE SYNTHESIS AND CHARACTERIZATION OF DENDRONIZED BIOMACROMOLECULES

By

Evan Burns, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

In Chemistry

Master of Science (2021) McMaster University

Chemistry Hamilton, Ontario, Canada

TITLE: The Synthesis and Characterization of Dendronized Biomacromolecules

AUTHOR: Evan Burns

BSc. (Chemistry)

McMaster University, Hamilton, Canada

SUPERVISOR: Professor Alex Adronov

NUMBER OF PAGES: xvii, 121

Abstract

Dendrimers are a unique class of polymer characterized by their structural perfection, monodisperse nature, and well defined, multi-valent periphery. These reasons, among others, make dendrimers an attractive polymer for use in biological systems, particularly in the field of polymer-protein therapeutics. While many therapeutics make use of a native enzyme, tethering a polymer to the therapeutic has many advantages such as increasing the lifetime of the therapeutic, and its overall efficacy. Polymer-protein therapeutics however must be selective to which biomolecules they interact with. While small molecule interactions are important to the regulation of the therapeutic, larger macromolecular interactions can often have a negative impact. It therefore becomes a challenge to construct polymer protein therapeutics that can selectively control these substrate interactions. The molecular sieving effect investigates this idea through the preparation of polymer-protein conjugates that can selectively interact with biomolecules of certain sizes.

While it has recently been shown that the molecular sieving effect can be achieved using high generation dendrimers that are quite large in size, these dendrimers are time consuming, and laborious to construct. In this work we sought out to further explore the molecular sieving effect using a series of novel linear-dendritic hybrid polymers. We hypothesized that appending a linear polymer to a lower generation dendrimer would help to prepare the larger polymeric constructs necessary for molecular sieving, in a more time- and labour-efficient manner. Furthermore, the addition of a linear polymer onto a dendrimer can have a beneficial effect the overall behaviour of the eventual conjugate.

Herein, we describe the synthesis of a library of linear-dendritic hybrid polymers and their conjugations to a model enzyme. This library consists of 9 novel linear-dendritic hybrid polymers, each varying in the generation of dendrimer used, and the size of linear polymer, PEG, appended

to the dendrimer's periphery. We first prepared these dendrimers using divergent synthesis approach, however, dendrimer core modifications post-PEGylation were unable to be performed due to complete core isolation within our dendrimers. We then developed a convergent synthesis approach to prepare these dendrimers which proved to be a better alternative as core modifications before PEGylation circumvented the issues we faced with the divergent approach. Each PEGylated dendrimer was functionalized to obtain a reactive DBCO moiety that would allow for simple conjugation to our model enzyme using click chemistry. Upon full characterization of each linear-dendritic hybrid polymer, select analogs were then conjugated to our model protein, α-chymotrypsin, which was functionalized with azide residues. SPAAC chemistry allowed for full conjugation to proceed quickly, and with minimal purification. After a full characterization of each conjugate, it was concluded that each conjugate was fully dendronized as intended.

Acknowledgements

I feel so lucky to have had the support of so many awesome people throughout my graduate studies. Completing a Master's degree is difficult enough on its own, but doing so amidst a global pandemic certainly brought up many new challenges. I definitely could not have done this alone, and am forever thankful for the great support network of friends, family, and colleagues who never failed to cheer me on along the way.

I first would like to give my sincerest gratitude to my supervisor, Professor Alex Adronov. I feel so fortunate to have had such a supportive, and knowledgeable supervisor throughout this process. Thank you for all of the insight you provided me over these past two years and for teaching me how to become a more detailed oriented scientist. I am appreciative how you took the time to make sure I was achieving at the level I needed to be, and for constantly pushing me to become a better graduate student, and researcher. I also want to thank my committee member Dr. Ryan Wylie for his insight and feedback throughout the progression of my degree.

A special thanks is given to Dr. Stuart McNelles, for all of the knowledge he provided me with while working together and for his patience with me when I first started grad school. Thank you for challenging my thinking and allowing me to become a better chemist in the process.

I want thank MCRMS staff, Kirk and Megan, for their assistance with mass spectrometry, the NMR staff, Bob and Hilary, for their assistance in running NMR experiments, and Matt Campea, for running my samples on DLS. Thank you to Elna Luckham at the Biointerfaces Institute for not only training me on the MALDI-MS instrument, but for being so helpful whenever I had questions and needed assistance when running my samples.

I would next like to thank the Adronov research group, both members of the past, and present. Thank you all for helping me with day-to-day lab issues, troubleshooting experiments,

and being there when I needed advice. I am particularly thankful for the friendships I have made with Billy, Dialia, Mohka, Ava, Mina, and Rena. Thank you all for entertaining me when I needed someone to talk to, and for making the lab a fun place to be. I also want to give a special shout-out to Billy for collaborating with me on this project during my final weeks here, and for taking it over once I leave, I wish you well on it.

I am so thankful for all of my friends that have supported me over the years, both in and out of school. Natalie, thank you for being my main support system throughout this entire process. I am so glad we got to experience grad school together and all the highs and lows that came with it. Thank you for always being there for me when I needed to complain about the most minor inconveniences, listening to me rant about the frustrations of grad school, and for lunch beers at the Phoenix. I truly believe every grad student needs a Natalie to help get them through the process. Rick, thank you for always being there for me when things became stressful. Thank you for showing me how to keep things in perspective, and how to keep my cool when times got tough. I am so fortunate to have had you by my side throughout this process. Eddy, I am so glad that grad school allowed us to reconnect. Thank you for always being a good listener, a good friend, and for teaching me how to keep a positive attitude along the way. To Sarah, Daniel, Victoria, Kathryn and Zach, thank you for being by my side since day one at McMaster and for all of the memories we have shared over the years.

Lastly, I want to thank my family for their constant love and support. To my parents, John and Rachel, I am so fortunate to have had you in my corner this whole time and I am forever grateful for your financial contributions to my education.

Table of Contents

List of Abbreviations

α-CT	α-Chymotrypsin
AcOH	Acetic Acid
ATRP	Atom Transfer Radical Polymerization
BHT	Butylated Hydroxytoluene
Bis-MPA	2,2-Bis(hydroxymethyl)propionic Acid
Boc	*tert*-Butyloxycarbamate
BSA	Bovine Serum Albumin
BT*p*NA	Benzoyl-L-Tyrosine p-Nitroanilide
Cbz	Carboxybenzyl Carbamate
CDI	N,N'-Carbonyldiimidazole
CuAAC	Copper-Catalyzed Azide-Alkyne Cycloaddition
DBCO	Dibenzoazacyclooctyne
DCM	Dichloromethane
DLS	Dynamic Light Scattering
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EPR	Enhanced Permeability and Retention
ESI-MS	Electrospray Ionization Mass Spectrometry
EtOAc	Ethyl Acetate
EtOH	Ethanol

FPE	Fluoride Promoted Esterification
FTIR	Fourier Transform Infrared Spectroscopy
G	Generation
LDA	Lithium Diisopropylamide
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionization - Time of Flight - Mass Spectrometry
MeCN	Acetonitrile
MeOH	Methanol
mPEG	Methoxypolyethylene Glycol
MS	Mass Spectrometry
MsCl	Methanesulfonyl Chloride
MWCO	Molecular Weight Cut Off
NHS	N-Hydroxysuccinimide
NMI	1-Methylimidazole
NMR	Nuclear Magnetic Resonance Spectroscopy
OMs	Mesylate
pCBMA	Poly(carboxybetaine methacrylate)
PEG	Polyethylene Glycol
POEGMA	Poly(oligoethyleneglycol) Methacrylate
ppm	Parts Per Million
RAFT	Reversible Addition Fragmentation Chain Transfer
SPAAC	Strain Promoted Azide-Alkyne Cycloaddition
TBABr	Tetrabutylammonium Bromide

TBAF	Tetrabutylammonium Fluoride
TCFH	*N*,*N*,*N*′,*N*′-Tetramethylchloroformamidinium Hexafluorophosphate
TCO	*trans*-Cyclooctene
TEA	Triethylamine
TEAF	Tetraethylammonium Fluoride
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran
TsOH	*para*-Toluenesulfonic Acid
UV-Vis	Ultraviolet Visible

List of Tables

List of Figures

List of Schemes

Chapter 1: Introduction

1.1 Polymer Architecture

Polymers can be found everywhere around us whether it be in nature, clothes, plastics, or even our own DNA. Polymer chemistry was first explored in the nineteenth century and involves the synthesis, characterization, and properties of polymers and other macromolecules.[1] Such polymers can involve natural polymers including proteins and sugars, or synthetic polymers including polyesters and polyolfeins. What makes polymers interesting is their differences in chemical structure, or architecture. These differences are what give the polymer their shape and help dictate their behaviour.

As shown in Figure 1.1, polymers can be made with a variety of different architectures each with their own special properties and characteristics. Linear polymers as shown on the left are the most common type of polymer and this type of architecture is seen in many commonly used items including rubbers, adhesives, and nylons. Graft and branched polymers on the other hand have unique structures as a result of their branching points. They have a higher concentration of terminal functional groups which gives them different properties than linear polymers.[2] This, along with their high degree of branching changes many properties of the polymer including its solubility, crystalinity, and hydrodynamic radius.[3,4]

A special type of branched polymer, called a dendrimer, is of particular interest due to its regular banching pattern and perfectly symmetrical shape, which differs from ordinary branched polymers. These chracteristics, among others, make dendrimers an attractive platform for a variety of applications and will therefore be the focus of the work presented in this thesis.

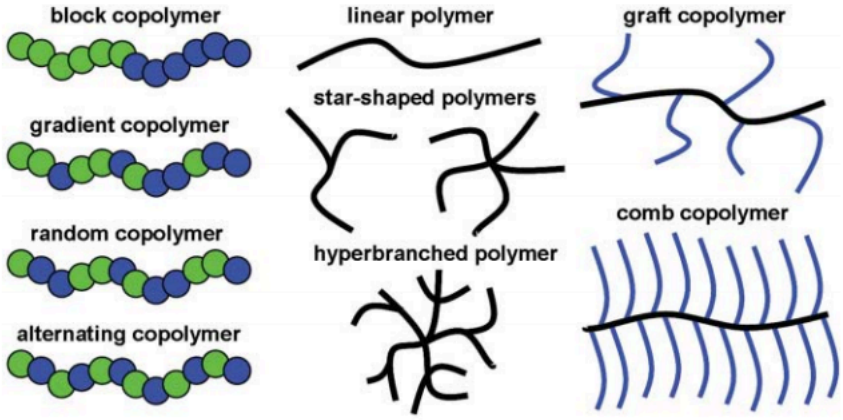


Figure 1.1. Overview of select polymer architectures.[5]

1.2 Introduction to Dendrimers

Dendrimers represent a unique macromolecular architecture that is optimally branched and structurally perfect. They are different from traditional polymers in that they are monodisperse and their branched nature forms tree-like structures rather than linear chains as illustrated in Figure 1.2. A dendron, also shown in Figure 1.2, is similar in structure to a dendrimer, however, has one key difference in that its core is chemically accessible. In other words, the core of a dendrimer is completely isolated and cannot be chemically manipulated, whereas the core of a dendron can. Despite this small difference, the terms dendron and dendrimer will be used interchangeably in this thesis.

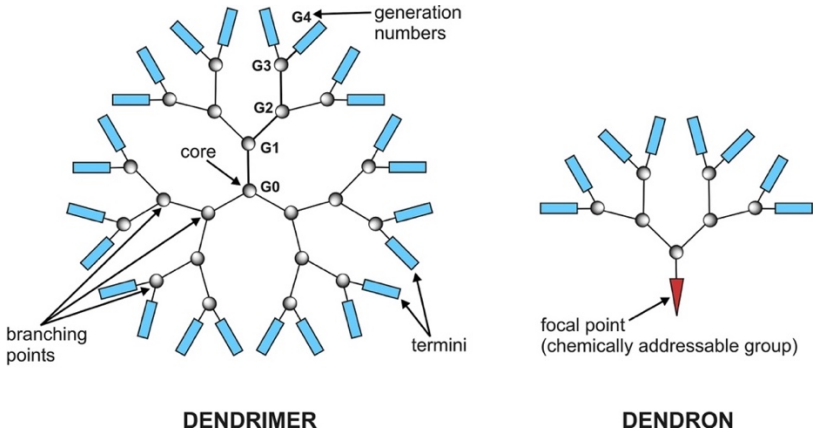
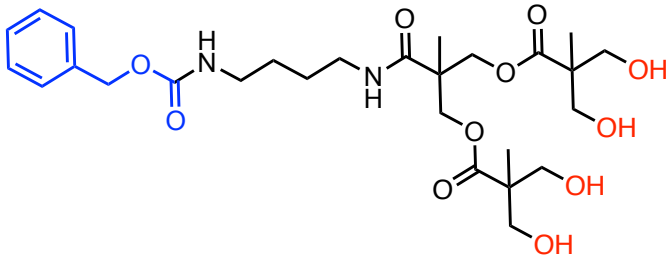


Figure 1.2. Illustration of dendritic structure.

1.2.1 Dendrimer Structure

Dendrimer structures are made of 3 main components- the core, the interior layers of branching units, and the periphery (or terminus), as illustrated in Figure 1.2. The core is located at the center of the molecule and is surrounded by the interior layers of branching units. Each layer is referred to as a generation, which is introduced in a stepwise manner and can be precisely characterized to ensure structural perfection. At higher generations the core is completely surrounded by the interior. The peripheral functionality, located at the dendrimer surface, can often dominate the properties of the dendrimer because it makes up roughly half of the dendrimer's mass.

When naming or classifying dendrimers it is common to refer to them by the structure at the core followed by the generation number, and then the peripheral functional group. For example, Cbz-G2-$(OH)_4$ shown in Figure 1.3 is used to classify a dendrimer that has a Cbz group at its core, is of second generation, and has 4 peripheral alcohol groups. This notation will be commonly used throughout the thesis.



Cbz-G2-$(OH)_4$

Figure 1.3. Cbz-G2-$(OH)_4$ dendrimer illustrating dendrimer nomenclature.

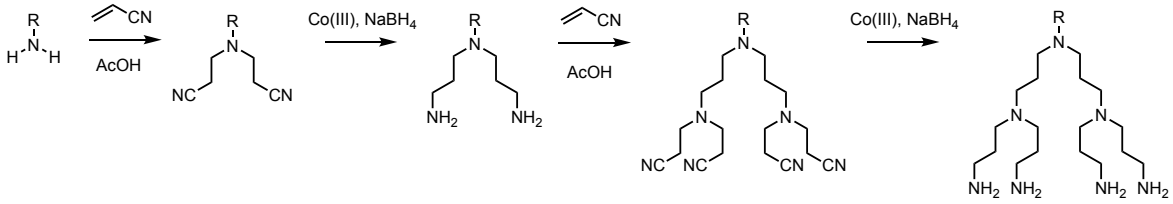
1.2.2 Properties of Dendrimers

Dendrimers are different from most polymers in that their theoretical dispersity is 1.0, which can be difficult to achieve in traditional polymer synthesis. This is made possible due to the

precise, and controlled addition of monomer at each step in the synthesis. While linear polymers adopt what is called a random coil conformation, dendrimers adopt a 3-dimensional globular morphology.[6] This is observed at higher generations and is typically first seen at the fourth generation.[7] Consequently, dendrimers do not exhibit a glass transition temperature due to their inability to intertwine with one another.[8] While in the globular formation at higher generations, the core is completely isolated from its environment as it is encapsulated by the periphery. When this was first realized it lead to the discovery of the "dendritic box" in which the dendrimer can host a smaller guest molecule within its interior.[9] While the periphery of a dendrimer is responsible for creating the "dendritic box", it also plays an important role in dictating its properties at higher generations as it constitutes the majority of the molecule's mass. A variety of properties such as polarity, solubility, and density can be controlled by modifying the dendrimer's periphery. Both the periphery and the core play an important role in dendrimer synthesis as they can both be chemically modified to obtain a desired functionality. It therefore comes as no surprise that dendrimers can lend themselves to a wide variety of applications[10] such as their use as sensors,[11] nuclear imaging agents,[12] drug delivery agents,[13] and host-guest chemistry.[14]

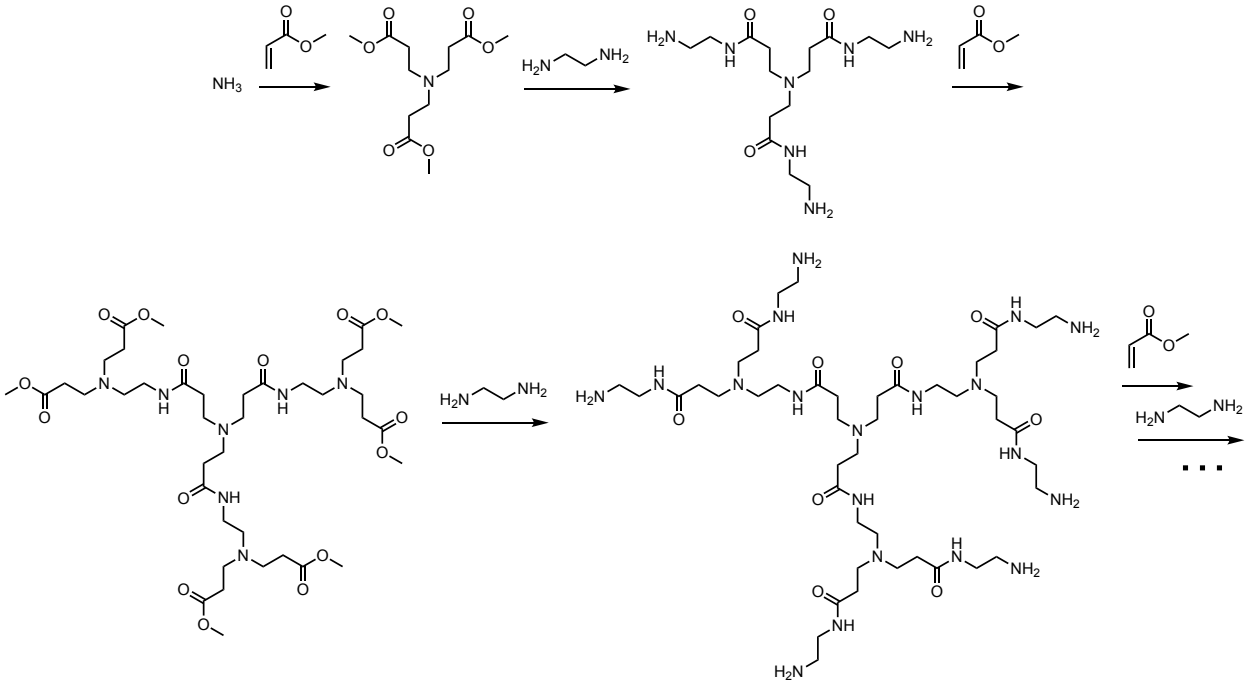
1.3 History of Dendrimers

Dendrimers were first reported in 1978 by Vogtle[15] and 1985 by Tomalia[16]. Vogtle's synthesis shown in Scheme 1.1 began with a Michael Addition between a primary amine and excess acrylonitrile to afford a bifunctional core. Subsequent reduction of the peripheral nitrile groups was performed using $NaBH_4$ and a Co(III) catalyst to form reactive amines. This stepwise process was repeated to make what he called "cascade polymers". These reactions however, were low yielding and susceptible to other side reactions, which limited the synthesis to lower molecular weight polymers which were not highly branched.[15,17]



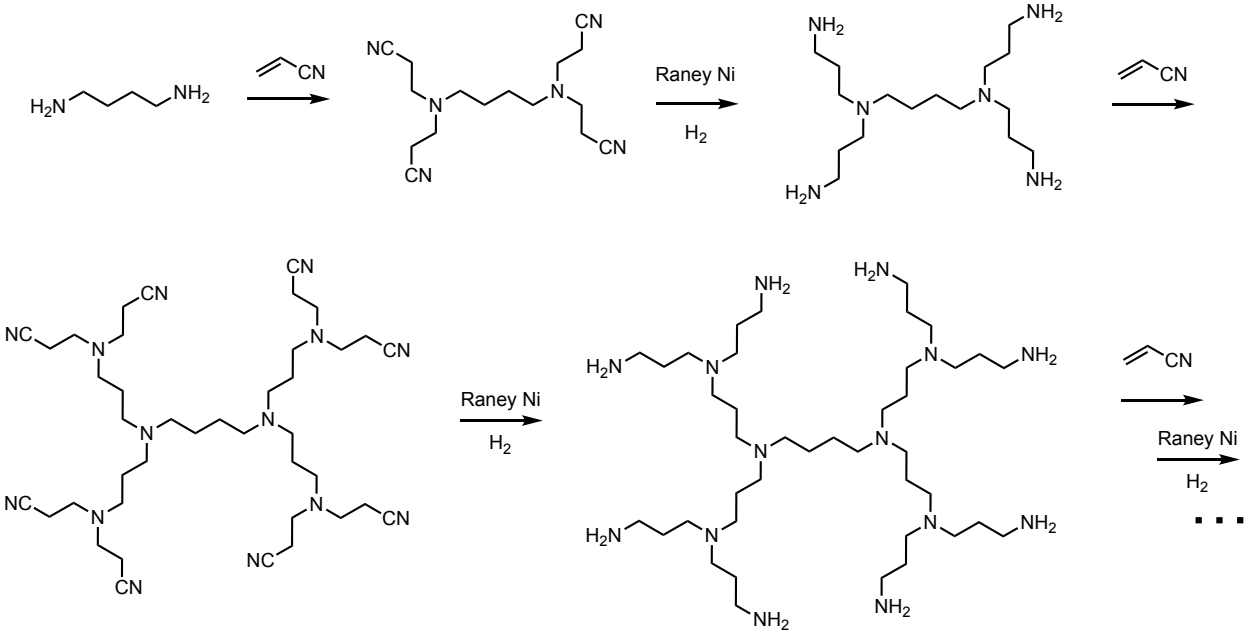
Scheme 1.1. Cascade polymer synthesis by Vogtle.

Tomalia built on the work of Vogtle by synthesizing what he initially called "starburst polymers", later referred to as dendrimers. In his synthesis, illustrated in Scheme 1.2, he combined ammonia with an excess of methyl acrylate in a Michael Addition to afford a trifunctional core. He grew his dendrimer by amidation of the peripheral esters with excess ethylenediamine to form a trifunctional polymer with amine groups at the periphery which could again react with methyl acrylate. These reactions were repeated stepwise to build a fifth generation dendrimer. This dendrimer, polyamidoamine, is one of the most common and commercially available dendrimers available due to its ease of preparation and ability to be prepared in higher generations.[16,17]



Scheme 1.2. Synthesis of polyamidoamine by Tomalia.

In 1993, Meijer took the pioneering work by Vogtle and optimized it to make poly(propylene imine) dendrimers, as Vogtle's "cascade polymers" were low yielding and susceptible to other side reactions. Meijer's work was very similar in execution to Vogtle's as he started with an amine core, 1,4-diaminobutane, which reacted with acrylonitrile in a Michael Addition. However, instead of reducing the nitrile group with Co(III) and $NaBH_4$, he used Raney Ni with H_2 as shown in Scheme 1.3. This series of reactions were then repeated stepwise to make poly(propylene imine). This synthesis was achieved in higher yields than Vogtle's initial synthesis and therefore made this dendrimer a widely available reagent commercially for use in research.[18,19]



Scheme 1.3. Synthesis of poly(propylene imine) by Meijer.

1.4 Dendrimer Synthesis

Dendrimers can be synthesized in two different ways as shown in Figure 1.4, using either what is called a divergent synthesis or convergent synthesis approach. Divergent synthesis involves building the dendrimer from the core outward, whereas convergent synthesis builds the

dendrimer from the periphery inward toward the core. While both strategies are practical and effective, each has their own advantages and disadvantages.

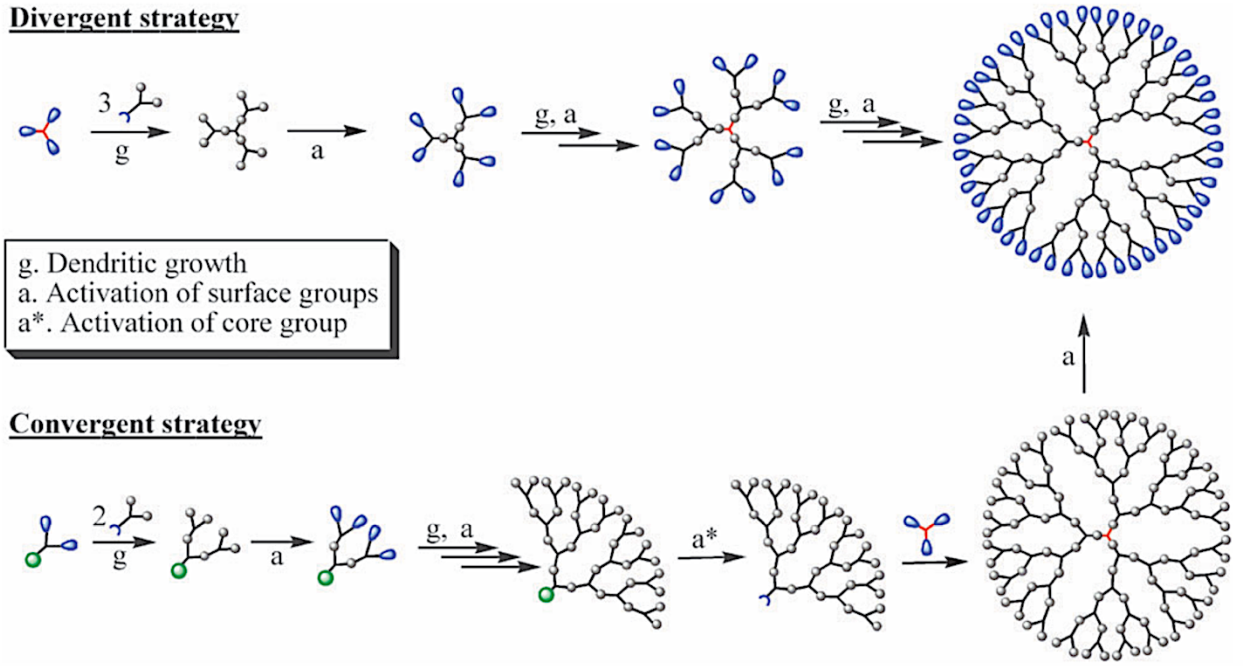


Figure 1.4. Illustration of the divergent and convergent methods for dendrimer synthesis.[20]

1.4.1 Divergent Synthesis

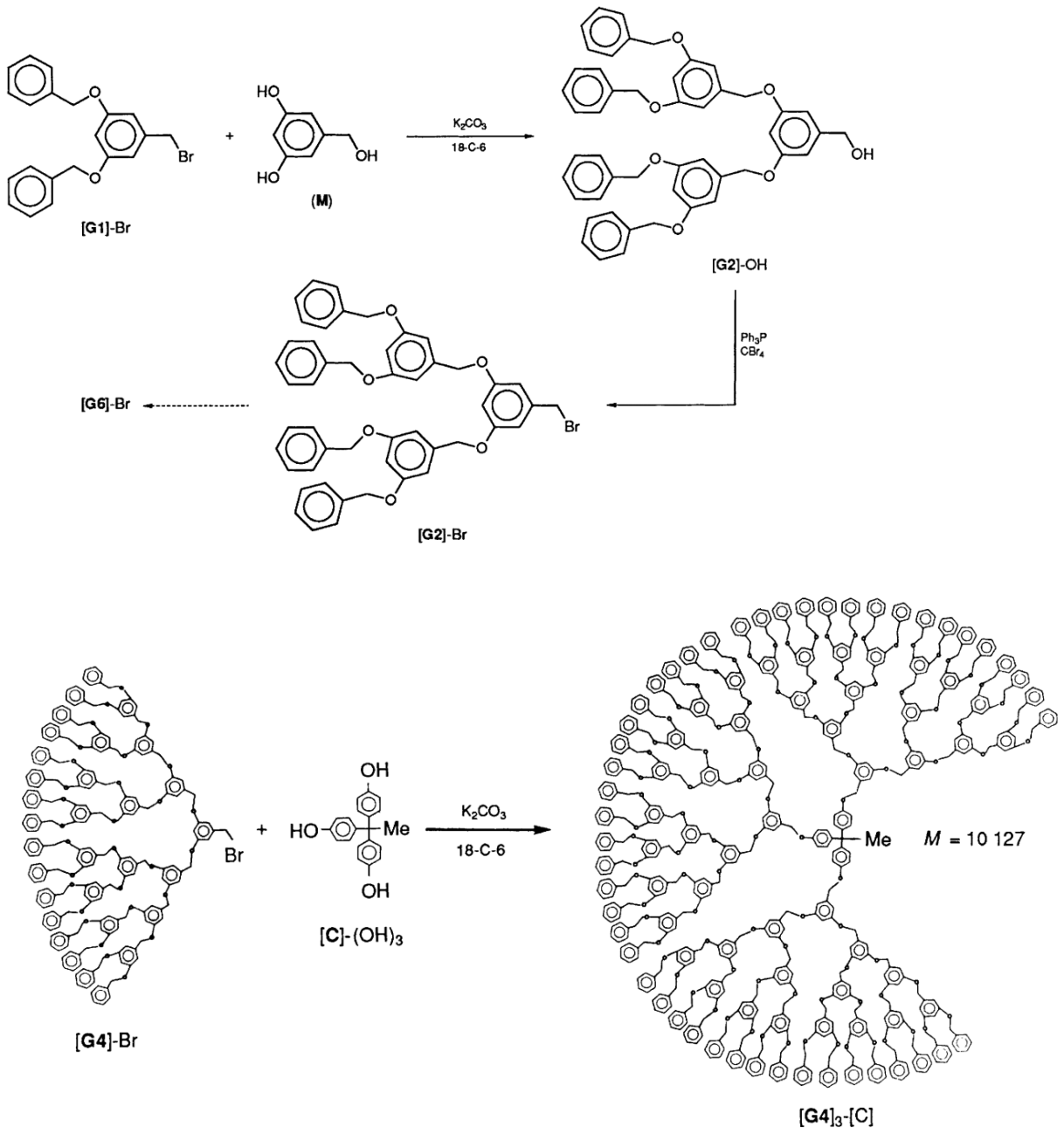
The dendrimers synthesized by Vogtle, Tomalia, and Meijer were all prepared using a divergent synthesis method. In this method, the core unit has some sort of functionality which allows reaction with a monomer unit in order to grow. Each monomer that is added has a protecting group on it to prevent further growth for that particular generation. This protecting group can then be removed in order to grow the dendrimer to the next generation. Monomers are often added in excess when growing a dendrimer to ensure full conversion to the next generation. Partial or almost full conversion of a dendrimer to the next generation is not ideal as this imperfection in structure will carry over to subsequent generations and cannot be corrected or purified later, increasing the product dispersity. The main advantage to using this approach is that with each generation that is added on, the amount of material doubles or can even grow exponentially depending on the number

of end groups present. Additionally, the monomer used is typically inexpensive, or easy to prepare. Disadvantages to this approach are that large amounts of monomer are typically needed for higher generation growth due to the number of end groups present. Moreover, if incomplete growth occurs, it is nearly impossible to purify as the partially functionalized material cannot separate from the fully functionalized product, especially at higher generations.[6,20,21]

1.4.2 Convergent Synthesis

The convergent synthesis approach was first reported in 1990 by Hawker and Fréchet as demonstrated in Scheme 1.4.[22] In this method, dendrons are first prepared in a stepwise fashion, and because they have a chemically addressable core they are added to the central core in the last step. In their work, Hawker and Fréchet first made polyether dendrons using 3,5-dihydroxybenzyl alcohol as their monomer. Reaction of the monomer with a benzylic bromide derivative in the presence of potassium carbonate and 18-crown-6 as demonstrated in the first step afforded a second generation dendron with an alcohol at its core. The benzyl alcohol was converted to another benzyl bromide using carbon tetrabromide and triphenylphosphine. These steps were repeated until the dendrons were appended to the core in the final step, yielding a sixth generation polyether dendrimer with a molecular weight of over 40 kDa.[22,23]

The convergent method is beneficial because the purification of each step is much easier than when using a divergent synthesis. This is because there is a vast difference in structure between the product and each of the dendron structures. For this reason, incomplete conversion to the next generation is not as problematic as when using the divergent approach. The disadvantages to this method are that reactions are lower yielding and steric interactions can make it difficult to append the high-generation dendrons to the core in the final synthetic step.[6,20]



Scheme 1.4. The first dendrimer prepared using a convergent synthesis by Hawker and Fréchet.[23]

1.5 Click Chemistry

Click chemistry involves reactions that are said to be wide in scope, high yielding, generate no by-products, or generate by-products that can removed through simple purification. Additionally, these reactions have broad solvent compatibility, are stereospecific, and have

products that are stable under physiological conditions.[24] There are many types of click reactions despite there being many criterion for a reaction to given this classification. Some common examples of click reactions include TCO-tetrazine, thiol-ene, CuAAC, and SPAAC. These click reactions have been used extensively in the literature to synthesize and functionalize dendrimers using either convergent or divergent syntheses.[17,20,25–37] Click chemistry can be advantageous to help grow and/or functionalize dendrimers, particularly at higher generations as non-click reactions involve high monomer loading, long reaction times, and cumbersome chromatographic separations.[38] Moreover, it is a great approach to ensure full functionalization of high generation dendrimers which is one of the main concerns when performing dendrimer synthesis.

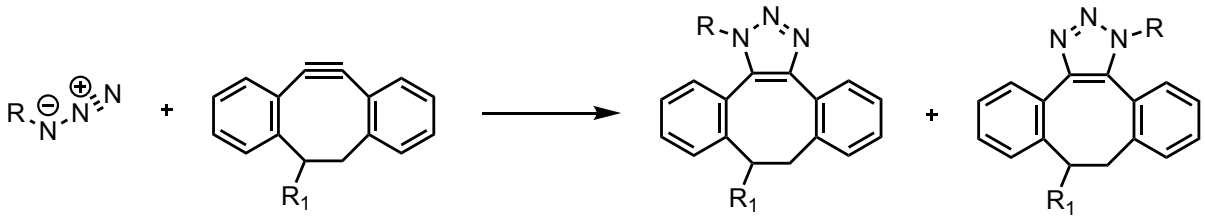
Malkoch recently showed that a variety of these click reactions can be used together in a one pot synthesis in which each reactant only reacted with its intended partner and no side reactions occurred. This one pot approach yielded a fourth generation dendrimer that was purified in one simple step. The dendrimer was isolated in an 89% yield and took less than 2.5 h of total reaction time to make.[28] Hawker also accomplished a similar feat in which they prepared a sixth generation dendrimer in a single day with the aid of click chemistry.[31]

Although these click reactions have been effective for the preparation of functional high generation dendrimers, each comes with its own limitations. For example, TCO-tetrazine chemistry can be problematic in that heat or light can cause isomerization of TCO to the *cis*-cyclooctene. This destroys the reactive component of the molecule and will no longer click if isomerization occurs. Thiol-ene chemistry has been reported to be more time consuming (relative to other click chemistries), especially when functionalizing high generation dendrimers, and early termination of thiol radicals can also destroy the reactive species.[27] CuAAC chemistry is not appropriate for biological applications as copper salts have been shown to be cytotoxic, even at

mM concentrations.[39] Finally, the SPAAC reaction can be limited in that cyclooctyne moieties can rearrange in the presence of acid and can decompose when heated.[40] Despite this, the SPAAC reaction is a great candidate for dendrimer synthesis as it does not require any excessive heating, external reagents, or catalysts. These characteristics also make it a better alternative for use in biological systems compared to other click reactions. Therefore, it will play an integral role in the synthesis of various dendritic structures in this thesis.

1.5.1 SPAAC Chemistry

The SPAAC reaction garnered a lot of popularity when first reported by Bertozzi for bioorthogonal chemistry in 2004.[41] The SPAAC reaction occurs between a cyclooctyne derivative and an azide group in a [3+2] cycloaddition as seen in Scheme 1.5.[42] Cyclooctynes exhibit a high degree of ring strain as they are the smallest all carbon cyclic alkynes that are both isolable and stable under ambient conditions.[43] Alleviation of this ring strain, along with the formation of an aromatic triazole are what drives this reaction irreversibly. This high degree of ring strain comes from the acetylene bond, which adopts bond angles of 163° instead of the ideal 180° bond angle of sp-hybridized carbon atoms.[42] This results in ring strain that amounts to ~18 kcal/mol,[44] significantly increasing reactivity.



Scheme 1.5. SPAAC reaction between an azide and cyclooctyne derivative.

The first cyclooctyne derivative was developed in 2004 by Bertozzi[41] and although novel, had relatively slow reaction kinetics (k_2 = 0.0012 M^{-1} s^{-1})[45], especially compared to the CuAAC reaction.[42] Consequently, a variety of cyclooctyne derivatives have been developed over the years,

each being modified to improve their reactivity. One way this was achieved was through the addition of electron withdrawing groups such as fluorine atoms, amide or carbonyl groups.[46]Another advancement to these derivatives included preparation of (di)benzoannulated cyclooctynes, such as the one in Figure 1.11. This modification increases the cyclooctyne ring strain through the addition of multiple sp^2 hybridized carbons adjacent to the acetylene carbons.[42] These developments, among others have increased the rate constant of cyclooctyne derivatives and SPAAC reaction kinetics by several orders of magnitude, including the most recent development in 2012 which has a second order rate constant of 4.0 M^{-1} s^{-1}.[45]

Although still slower than the CuAAC reaction[47], the SPAAC reaction has garnered a lot of attention for uses in biological systems, especially in the field of bioconjugation due to its ease of operation, biorthogonality, and use of non-toxic materials.[46,48,49]

1.6 Polymer Protein Conjugates

As their name suggests, polymer protein conjugates are a class of biohybrid macromolecules that are made by covalently linking a synthetic polymer to a polypeptide.[50] These materials are extremely advantageous as the polymers linked can help stabilize the proteins, give them functionality, and/or enhance their activity.[50–52] The first polymer protein conjugate was prepared in 1977 by Abuchowski where they coupled monomethoxy-PEG to BSA to investigate the effect of PEG on the immune response, relative to the native protein. They found that the BSA-PEG conjugates both increased blood circulation times, and displayed a lower immunogenic response in animal models relative to the native protein.[53] These ground-breaking findings sparked a great deal of interest in the field of polymer protein conjugates, with particular attention aimed at the use of PEG conjugates.[51] As a result, there are currently numerous polymer protein

conjugates used extensively in research with a primary focus being on their use as therapeutics.[50,51,54–56]

1.6.1 Preparation of Polymer Protein Conjugates

Preparation of polymer protein conjugates commonly occurs in one of three ways; grafting to, grafting from, or grafting through, as depicted in Figure 1.5. Grafting to involves coupling functionalized polymers to specific functional groups such as thiols, amines, or carboxylic acids that are on the surface of the protein. This method often makes use of click chemistry as these reactions are highly selective, and able to proceed under mild conditions. The grafting from method involves the polymerization of a monomer from the surface of the protein through the use of a macroinitiator, which is an initiator bound to the protein. RAFT or ATRP chemistry are most commonly used when growing the polymer chain from the macroinitiator. Dispersity of the polymer can be controlled, and this method can be done in biologically relevant conditions, which also makes the grafting from method attractive. The grafting through method is the least common and most complex. It involves first linking monomer functional groups to the protein to form what is called a macromonomer. The monomer of interest can then be grafted onto the macromonomer through copolymerization. This method can be problematic due to unwanted side reactions with other functional groups on the polypeptide. To circumvent this, extra steps are needed to protect these groups, which make this method more difficult and time consuming.[50,54,55]

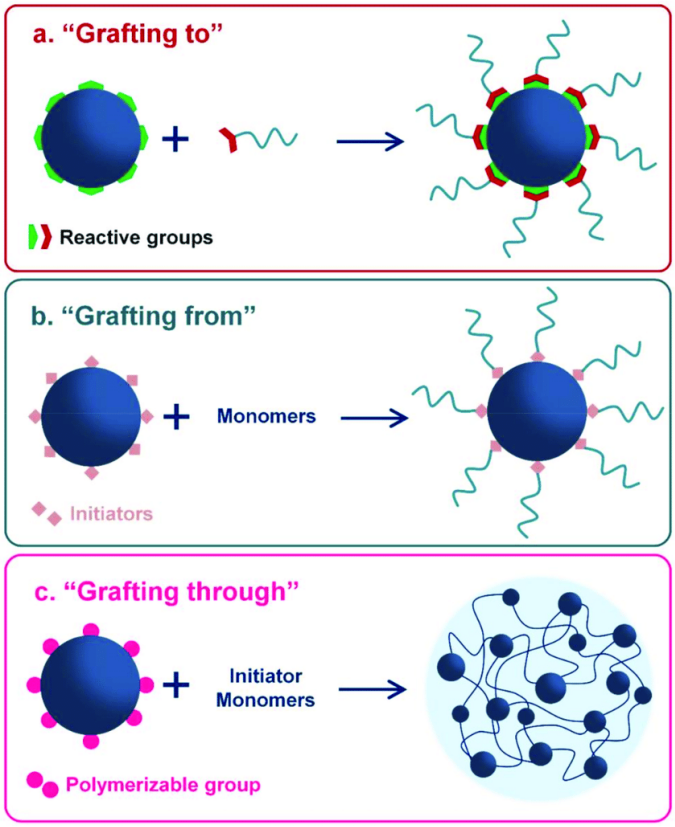


Figure 1.5. Methods of preparing polymer-protein conjugates.[57]

1.6.2 Uses as Therapeutic Agents

Although native proteins such as antibodies and enzymes have been proven to serve as therapeutic agents for many illnesses, there are many downfalls to using these native, unconjugated proteins, such as their short blood circulation times and non-specific toxicity.[58–60] Even more important however, is that they exhibit a degree of instability *in vivo* as they are susceptible to denaturation when exposed to changes in temperature and pH. Furthermore, the behaviours of these proteins can sometimes be unpredictable *in vivo* and protease degradation will easily impair its function as a therapeutic agent.[50] All of these issues can be circumvented through the use of polymer protein conjugates as therapeutic agents, as conjugation of a polymer to a protein can have a beneficial effect on the behaviour of the protein all while still maintaining its therapeutic abilities.[50,60]

Conjugating polymers to therapeutic proteins has several advantages, one of the most important being that they reduce immunogenicity of the protein.[61] Additionally, they can help improve the solubility, pharmacokinetics, and controlled delivery of the therapeutic.[62] Conjugation can also substantially increase its molecular weight which reduces renal clearance and prolongs the blood circulation time which can enhance the EPR effect.[60] These polymers can help improve the lifetime and stability of protein therapeutics as they can prevent denaturation and degradation of the protein by effectively shielding it.[50] Polymers can have more predictable behaviour in terms of their response to stimuli such as changes in temperature or pH. As such, they can be carefully constructed to exhibit stability in response to these stimuli which makes them better suited as front line defenses *in vivo* compared to their protein counterparts.[50,63]

There are currently many marketed polymer protein conjugates that function as therapeutics for a variety of infections and illnesses. Although many different polymers can be used in these therapeutics, majority of these make use of one of the simplest, yet effective polymers on the market, PEG.[62]

1.6.3 PEGylated Conjugates

After their first use in 1977 by Abuchowski, PEGylated conjugates have garnered a lot of attention for use in polymer protein therapeutics.[51] These conjugates have significantly improved the treatment of many chronic diseases including leukemia, hepatitis C, and rheumatoid arthritis.[64] Compared to other polymers, PEG has a lot of desirable qualities for use in biological systems including being water soluble, non-toxic, low-bio-fouling, and a thermal stabilizing agent.[51,55,65] Additionally, PEG is regarded as safe by the FDA and is able to increase bio-circulation times when used *in vivo*. It does this by behaving like a “stealth” molecule and avoids being recognized by the immune system which would mark it for opsonization and eventual phagocytosis.[51]

PEGylated conjugates are protected by the outer hydrophilic layer that is created by the PEG polymer. A hydration shell is formed when water binds to the PEG and this shell is what effectively prevents any protein binding and reduces the overall immune response.[51] The pioneering work by Abuchowski illustrated this notion by preparing PEGylated bovine liver catalase which effectively increased the blood circulation by 10-fold, compared to the native protein.[66]

PEG however does have a few drawbacks including being non-biodegradable[55] and the cause of continued allergic reactions in some patients.[67] More importantly, repeated dosages have been shown to form anti-PEG antibodies, which over time increase clearance rates of PEGylated conjugates thus reducing their efficacy.[68] This has prompted researchers to investigate alternative polymers for protein conjugation which could possibly circumvent these issues.[51]

Furthermore, PEGylation of protein therapeutics can also come at a cost of reduced protein activity. For example, increasing the PEG chain length and grafting density on conjugates can increase blood circulation times and lower immunogenicity, but also simultaneously begins to block off the enzyme active site. If this is done to a certain extent, it can entirely block off the active site of the conjugated enzyme, completely diminishing its activity.[64,69] It has also been proven that PEGylation influences the kinetics of substrate binding. PEG conjugation decreases both the catalytic turnover and substrate affinity of the enzyme and this effect becomes more pronounced as PEGylation increases.[70] Lastly, PEG can also diminish enzymatic activity through binding with hydrophobic pockets within the enzyme active site rendering it inaccessible.[71] It therefore becomes a challenge to create PEGylated conjugates that have PEG chains that are large enough to increase blood circulation times and decrease immune responses, but short enough to still retain most, or partial enzymatic activity.

1.7 PEGylated Dendrimers

Similar to other polymer protein therapeutics, PEGylation of dendrimers has been shown to improve their pharmacokinetic properties as proven with biodistribution studies and the EPR effect. These properties, namely blood circulation times can be optimized through controlling the PEG chain length and its grafting density to the dendrimer.[72] In other words, circulation times of PEGylated dendrimers can be controlled by amount of branching and the overall hydrodynamic volume. For example, Gillies demonstrated that a G3 dendrimer with 8 x 5 kDa PEG chains has a longer circulation time compared to a G1 dendrimer with 2 x 20 kDa PEG chains, by 20-fold, even though they are similar in molecular weight. Contrarily, a G1 dendrimer with 2 x 10 kDa PEG chains and a G1 dendrimer with 2 x 20 kDa PEG chains have very similar circulation times despite their vast differences in molecular weight. These results demonstrate that highly branched dendrimers exhibit increased circulation times due to their decreased flexibility and lack of reptative mobility that slows their passage through renal pores as illustrated in Figure 1.6. Dendrimers with a low degree of branching more resemble linear polymers and therefore have less structural rigidity. This allows them to more easily diffuse through renal pores despite differences in molecular weight.[73] A similar study by Fréchet also illustrated how dendrimers of similar molecular weight can exhibit vast differences in blood half-life based on the grafting density and length of PEG chains appended. Their results corroborate those from Gillies in that dendrimers with fewer, but higher molecular weight PEG chains displayed a more rapid clearance compared to dendrimers with more, but lower molecular weight PEG chains, despite being similar in molecular weight.[74]

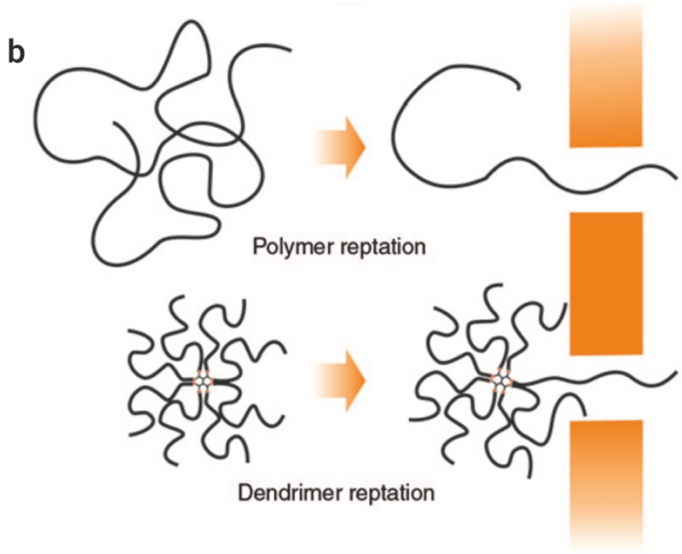


Figure 1.6. Effect of polymer architecture on renal filtration.[75]

In addition to improving the pharmacokinetic properties of the dendrimer, PEGylation can also protect the inner dendron structure from quick degradation or hydrolysis. Zhang and colleagues demonstrated that PEGylation of a polyester dendrimer slowed down the depolymerization process of the inner dendritic backbone in aqueous conditions. A G2 dendrimer with twelve PEG-500 chains grafted to the periphery through ester linkages began to degrade at physiological pH and temperature after three days. Full degradation of the dendritic backbone took just over two months.[76] Other studies using non-PEGylated analogs of the same dendrimers have shown that degradation at physiological pH and temperature can begin after just a few hours, and full degradation occurs after a few weeks.[77,78] These studies show that addition of PEG to the periphery of a polyester dendrimer can effectively close off and protect the ester linkages present in the dendritic backbone which effectively slows down their hydrolysis and degradation process.[76] It is therefore expected that this trend would remain consistent when used *in vivo*, thus making PEGylated dendrimers an even more attractive polymer for polymer-protein therapeutics.

PEGylated dendrimers can be referred to as what is called a “linear-dendritic hybrid polymer”. This is a special type of macromolecule composed of a polymeric fragments with two dissimilar architectures: a linear chain, and a dendritic moiety.[79] PEGylated dendrimers, or PEGylated linear-dendritic hybrid polymers can be prepared in numerous ways by exploiting the various terminal functional groups that PEG can exhibit. For example, hydroxy, carboxy, amine, thiol, or azide terminated PEG are some functionalized PEG derivatives that can be prepared or purchased commercially. Nucleophilic acyl substitution reactions have been a common way to achieve PEGylation through the aid of carbonate[80–82], carboxylic acid[83–85] or other carbonyl groups[86–88] reacting with the appropriate PEG derivative. Although effective, these reactions can take time and frequently exhibit extensive purification. Partial conversion of the dendrimers also becomes a concern due to steric crowding at the periphery at higher generations. These problems have been circumvented with the aid of click chemistry as these reactions are typically quicker, have simpler purification methods, and ensure full functionalization due to more compatible reaction partners.[24] SPAAC, CuAAC and thiol-ene reactions have all been previously reported for dendrimer PEGylation, particularly for the PEGylation of high generation dendrimers.[27,30,88–91] These click reactions pose as viable methods for PEGylation over other click reactions as the functional groups needed for these reactions can easily be incorporated to both the periphery of the dendrimer and the terminus of the PEG chain.

1.8 Molecular Sieving

Molecular sieving is a relatively new and emerging concept within the field of polymer-protein conjugates. It has involved appending polymers of various architecture to specific sites on the surface of an enzyme to create a polymeric shell. If this shell is properly designed, small molecules can pass through it to access the enzyme’s active site, but macromolecules beyond a

certain size such as proteins and antibodies can be blocked.[92,93] Despite the field of molecular sieving only being recently discovered and not yet used clinically, it poses as an important concept within polymer-protein therapeutics. Conjugates used in therapy for example, need to reject protein-antibody interactions and protease-mediated hydrolysis, all while allowing smaller, vital proteins to interact with their substrates.[94] For example, consider L-asparaginase, an enzyme used to treat acute lymphoblastic leukemia. When polymers such as PEG are grafted to its surface, the conjugate should reject proteins from the immune system while simultaneously allowing the small molecule L-asparagine to the enzyme's active site.[92]

Pioneering work in this field utilized graft polymers[92,95] to achieve this affect, whereas recent developments have been able to illustrate the same effect with more complex polymer structures, namely dendrimers.[93]

1.8.1 Early Work Using Graft Polymers

The molecular sieving effect was first reported by Gauthier in 2013.[92] In their work they prepared a library of POEGMA graft polymers with varying backbone and side chain lengths using ATRP chemistry and the grafting from approach. These polymers were then appended to α-chymotrypsin to form various polymer-protein conjugates with unique combinations of grafting density. It was observed that based on the grafting density, POEGMA would form different favourable globular conformations around the enzyme active site which would exhibit the molecular sieving effect. As observed in Figure 1.7, the brush-ellipsoid conformation produced small interstitial spaces which allowed smaller molecules to penetrate their way through the globular polymer backbone, all while rejecting larger molecules, thus illustrating the sieving effect. Other forms of POEGMA were ineffective, or not as effective. This pioneering work showed that a globular polymeric shell with abundant surface coverage is required for sieving. Although the

length and size of the polymer are important to achieve molecular sieving, grafting density is the most important parameter to effectively shield the enzyme.[92]

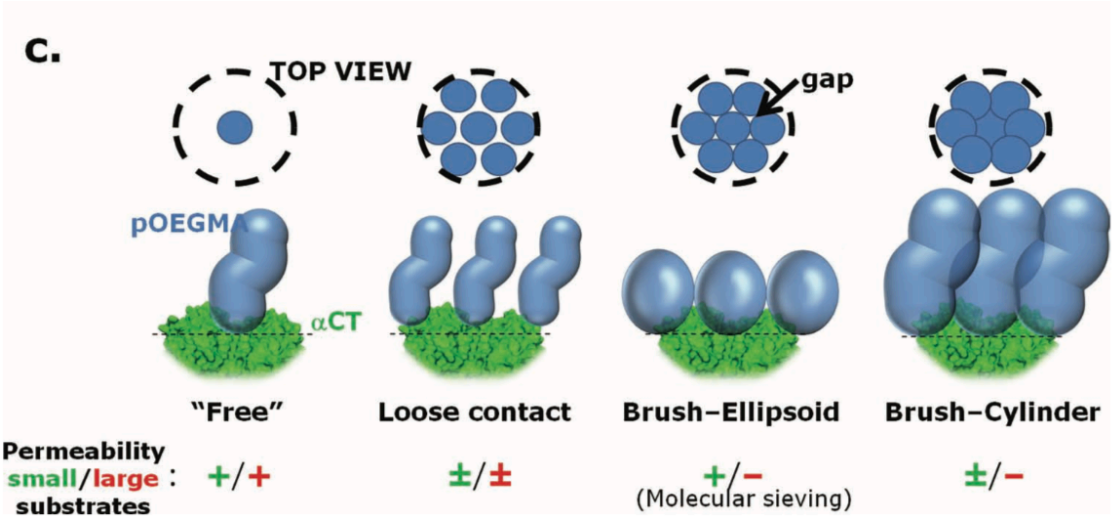


Figure 1.7. POEGMA conformations as a result of grafting density.[92]

Years later, the works of Russell demonstrated similar results to Gauthier by also using graft polymers. In their work, ATRP chemistry was used to decorate avidin with pCBMA polymers, using the grafting from method. These polymers were prepared with varying backbone lengths and appended to avidin with variable grafting densities. In addition to a regular ATRP initiator, they used a double-headed ATRP initiator when growing the polymer which virtually doubled its grafting density as seen in Figure 1.8. They again observed that the rate of sieving is strongly dependent on the grafting density of the polymer and is less dependent on the length or size of the polymer. They were also able to demonstrate that grafting density has a pronounced effect on the rate of substrate binding. Kinetic studies revealed that high density avidin-pCBMA conjugates prepared from the single headed initiator had a 2-fold decrease in small molecule substrate binding rate compared to a lower density conjugate also prepared from the single headed initiator. Furthermore, doubling the grafting density on the high-density conjugates using the double-headed initiator caused a 10-fold decrease in substrate binding rate when compared to the

low density conjugates made with a single headed initiator. This study was the first reported molecular sieving study in which the rates of substrate binding were thoroughly examined and quantified.[94,96]

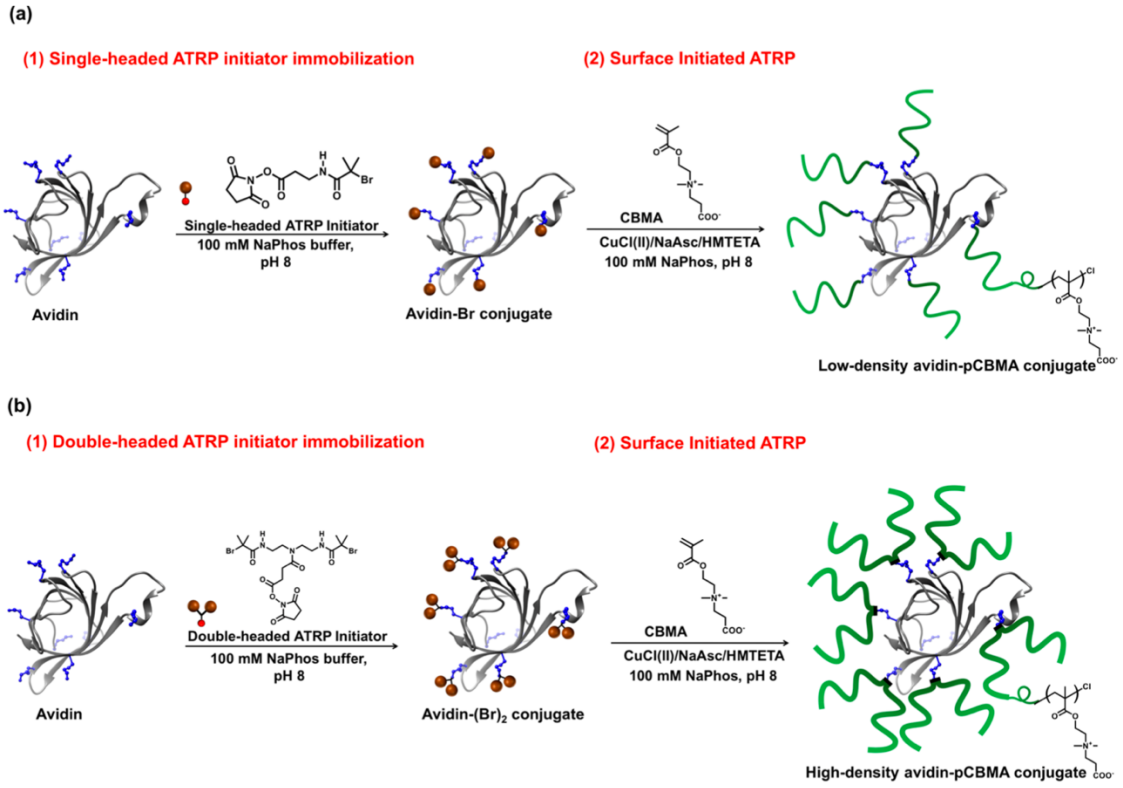


Figure 1.8. Synthesis of avidin-pCBMA conjugates a) low grafting density using a single headed ATRP initiator b) high grafting density using a double headed ATRP initiator.[94]

1.8.2 Recent Advances Using Dendrimers

Our group has recently extended this work by using dendrimers to achieve the molecular sieving effect shown in Figure 1.9. In this work, dendrimers of generations 1 through 8 were synthesized and their cores were modified to obtain a cyclooctyne moiety. These dendrimers were appended to azide decorated α-chymotrypsin through the use of SPAAC chemistry to form a variety of dendrimer-enzyme conjugates. These conjugates were then tested to see which generation(s) of dendrimer were able to achieve this sieving effect. It was shown that all of the

conjugates allowed a small molecule substrate, BT*p*NA to pass through the globular dendritic shell to the active site of α-chymotrypsin. However, generation 7 and 8 dendrimer-enzyme conjugates rejected larger substrates BSA and anti-α-chymotrypsin from the enzyme's active site, therefore demonstrating the molecular sieving effect. It was concluded that sieving is achieved using higher generation dendrimers because their larger, globular architecture effectively creates a larger polymeric shell that shields the enzyme active site. This work shows that these dendrimer-enzyme conjugates require a critical size, or generation, to achieve molecular sieving.[93]

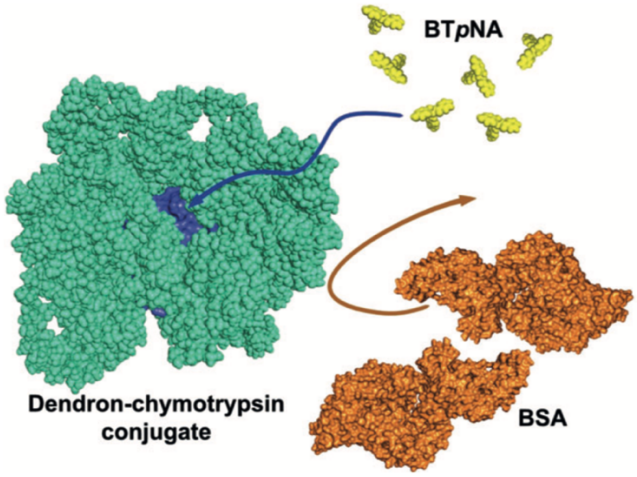


Figure 1.9. Diagrammatic representation of the molecular sieving effect using a dendronized conjugate.[93]

1.8.3 Advantages and Limitations of Dendrimers

Although conjugates prepared from graft and linear polymers have proven to be effective in molecular sieving, there are some limitations to their use in therapeutic applications when compared to dendronized conjugates. For example, dendrimers have a well-defined, multi-valent periphery which allows for precise, controlled addition of specific groups such as drug molecules, or targeting vectors. Additionally, linear and branched polymers are often polydisperse which makes it difficult to provide reproducible pharmacokinetic behaviour. Furthermore, the use of

monodisperse dendrimers ensures full and uniform coverage of the protein when preparing conjugates, thus ensuring a more consistent polymeric shell around the protein.[74-76]

While dendrimers are preferred over linear and branched polymers for bio-conjugation and molecular sieving, a major limitation in their use comes with their preparation. Construction of higher generation dendrimers is laborious, and can often take multiple days, or even weeks to prepare. Furthermore, when preparing higher generation dendrimers, convergent syntheses are typically lower yielding, and divergent syntheses require high monomer loading and much more precision. When considering all of these limitations together, the preparation of high generation dendrimers can prove to be a very time consuming and arduous task.

1.9 Objectives

The general goal of this thesis is to continue to focus on further exploring the use of dendrimers to achieve the molecular sieving effect. As previously mentioned, preparing dendrimers of higher generations can be time consuming and laborious. We have recently shown that the molecular sieving effect can successfully occur using a G7 or G8 bis-MPA dendrimer that is conjugated to α-chymotrypsin.[93] Rather than taking the time to make these high generation dendrimers we are interested in preparing a series of linear-dendritic hybrid polymers. We are interested in preparing lower generation dendrimers and then PEGylating them to essentially mimic the size of the higher ones. These PEGylated dendrimers can be used to prepare polymer protein conjugates that can be tested for their molecular sieving abilities as illustrated in Figure 1.10. PEGylation of these dendrimers not only helps to build larger dendrimer constructs quickly and more efficiently, but PEG is also of interest in biological applications and in the field of polymer protein therapeutics. As previously discussed, PEGylation helps to increase blood circulation times and decrease immunogenicity of various biomolecules. Moreover, PEG is low-

biofouling, water soluble, and exhibits low-toxicity, which make it an even more attractive polymer for conjugation. Therefore, investigation of the molecular sieving abilities of PEGylated conjugates will hopefully prove to be of greater benefit in a clinical setting compared conjugates that are not.

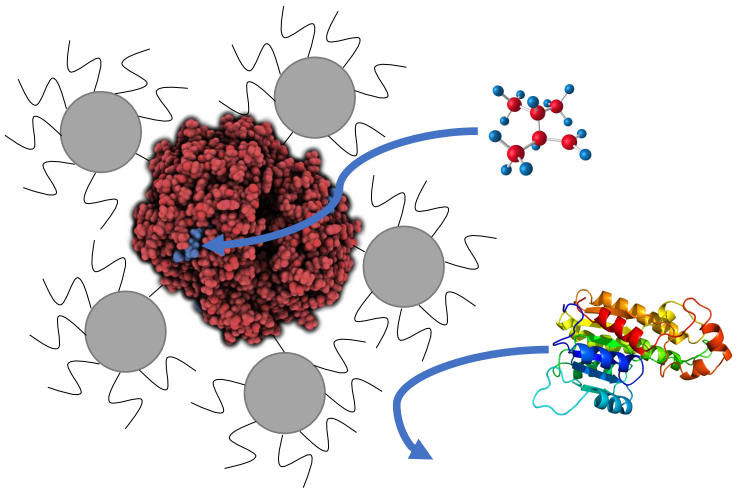


Figure 1.10. Illustration of the molecular sieving effect using a PEGylated dendronized conjugate.

More specifically, we plan to prepare G3-G5 bis-MPA dendrimers and then PEGylate their peripheries with PEG-350, PEG-1000, and PEG-2000 chains. This will form a library of 9 dendrimers each varying in generation and molecular weight PEG chain grafted to the periphery. We have previously shown that the non-PEGylated G3-G5 bis-MPA dendrimers do not exhibit sieving abilities but we hypothesize that some of the PEGylated analogs will. PEGylation will increase the dendrimer's hydrodynamic volume and will create a larger polymeric shell that will hopefully provide more surface coverage of the enzyme. Upon preparation of these dendrimers containing a DBCO core, they can be conjugated to azide functionalized α-chymotrypsin using SPAAC chemistry and the "grafting to" approach for bioconjugation. After fully characterizing

each conjugate to ensure that all azide residues have been clicked with a DBCO core PEGylated dendrimer, the dendrimer enzyme conjugates can undergo sieving studies.

In Chapter 2 we investigate the synthesis of these PEGylated dendrimers using a divergent synthesis approach. PEGylation using this method is achieved rather quickly, however, we unexpectedly discover that the core of the dendrimers becomes completely isolated and cannot be chemically manipulated to obtain a DBCO moiety for enzyme conjugation. We attempt to resolve this issue in Chapter 3 by using a convergent approach in which we modify our core before PEGylation, rather than after. Chapter 3 explores the success of this convergent approach as well as the full synthesis and characterization of the DBCO-core, PEGylated dendrimer library. Finally, in Chapter 4 we look at the preparation and full characterization of select dendrimer enzyme conjugates.

Chapter 2 - Dendrimer PEGylation Using A Divergent Approach

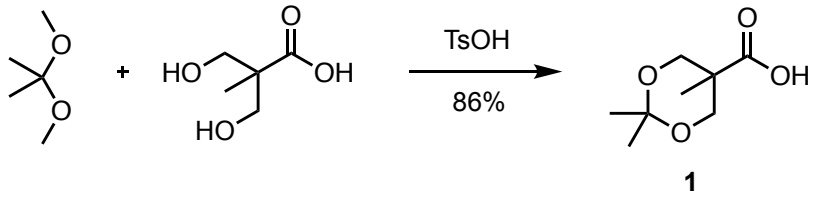
2.1 Overview

In this chapter, we explore a divergent synthesis approach to prepare our library of G3-G5 PEGylated dendrimers. This first involved preparing a series of bis-MPA dendrimers with a Cbz protected core up to the fifth generation. In order to ensure simple, and quick dendrimer PEGylation we planned to use the SPAAC reaction so that the most difficult synthetic step would become the easiest. To execute this strategy, the periphery of the G3-G5 dendrimers were first functionalized with azide groups which serve as reactive handles for PEGylation. Next, a series of amidated DBCO functionalized PEG chains with varying molecular weights were synthesized and these PEG chains were easily clicked onto the periphery of each azide functionalized dendrimer. Upon dendrimer PEGylation, core removal for the G3 analogs was attempted. We encountered an unexpected result in that only the core of the G3 dendrimer bearing PEG-350 chains was able to be removed. Multiple attempts at removing cores of the PEG-1000 and PEG-2000 G3 analogs were unsuccessful. Consequently, efforts to deprotect the cores of G4 and G5 PEGylated dendrimers were not carried out. Since the cores were unable to be deprotected, they consequently could not be modified to obtain a DBCO moiety for later conjugation with the model enzyme. This challenge renders the approach presented here incapable of achieving our goals, requiring an alternative strategy.

2.2 Synthesis of Azide Functionalized Bis-MPA Dendrimers

The first step in preparing the bis-MPA dendrimers was to synthesize a monomer. The monomer, bis-MPA-acetonide, **1**, was prepared by reaction between 2,2-

bis(hydroxymethyl)propionic acid, 2,2-dimethoxypropane, and an acid catalyst as shown in Scheme 2.1.

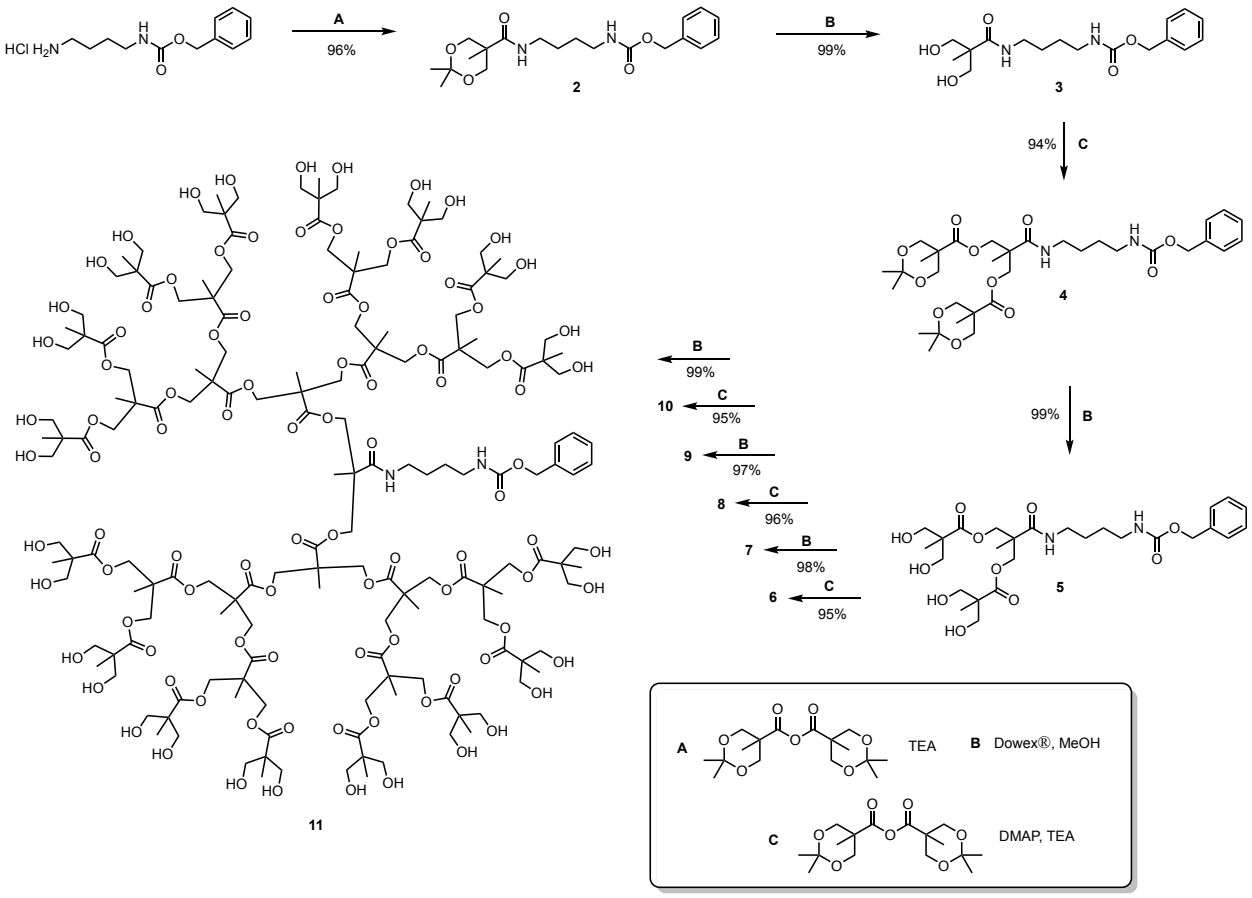


1

Scheme 2.1. Synthesis of bis-MPA-acetonide.

Upon preparation of **1**, dendrimers of generation 1 to 5 were synthesized starting from *N*-Z-1,4-butanediamine hydrochloride. As illustrated in Scheme 2.2, dendrimer synthesis began with amidation between *N*-Z-1,4-butanediamine hydrochloride and the anhydride of bis-MPA-acetonide to form the first generation acetonide protected dendrimer Cbz-G1-$(acet)_1$. It should be noted that the anhydride of bis-MPA-acetonide was made first through reaction of **1** with EDC and used directly in a one pot synthesis without purification for all steps shown in Scheme 2.2.

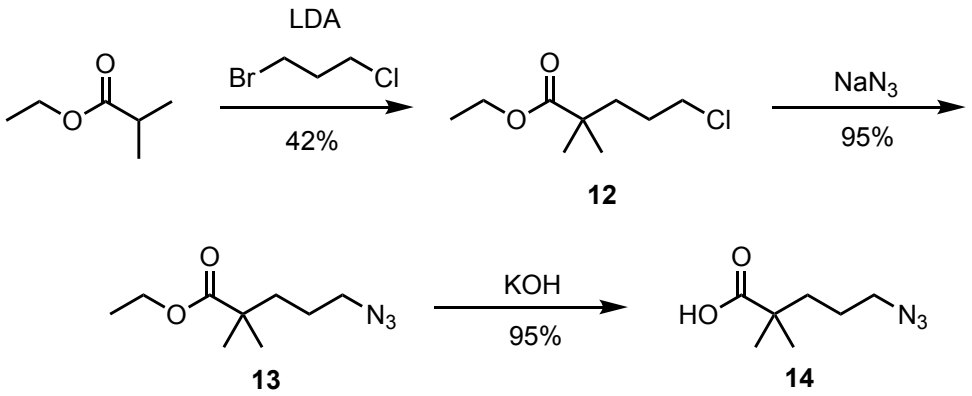
The following step to activate the dendrimer periphery was achieved using Dowex, an acidic ion exchange resin, which hydrolyzes the acetal protecting group with the help of MeOH to afford the first generation deprotected dendrimer Cbz-G1-$(OH)_2$. A Steglich esterification using the anhydride of bis-MPA-acetonide was used to grow the dendrimer to the second generation, followed by deprotection using Dowex. This series of growth and deprotection reactions was repeated as shown below until the fifth generation. All dendrimers were prepared in yields upwards of 95%.



Scheme 2.2. Synthesis of bis-MPA dendrimers of generations 1-5.

As previously mentioned, the approach to using the SPAAC reaction for divergent PEGylation involves functionalizing the periphery of the dendrimers with azide groups that can easily react with DBCO functionalized PEG chains. It is important to keep in mind that any group added onto our dendrimers must not be prone to hydrolysis as this will ruin the dendrimer's structural perfection. Functionalizing the periphery of our hydroxylated dendrimers can be accomplished using a carboxylic acid to form an ester linkage. However, this linkage must be protected as esters can be easily hydrolyzed. We opted to use neopentyl linkers to protect our esters from hydrolysis as work by our group has already focused on their use. Scheme 2.3 outlines a synthetic approach designed by our group to prepare a linker **14** which exhibits three important functionalities which are vital to preparing these functionalized dendrimers. The first being the

azide group which will be responsible for clicking with our DBCO functionalized PEG chains for dendrimer PEGylation. The next being the terminal carboxylic acid group which will be used in the next section for appending this small molecule onto the periphery of our dendrimers. The final important feature of this small molecule is the 2 methyl groups that are appended to the α carbon. These will prevent reaction of the carbonyl group with any water or other nucleophilic species which could hydrolyze the ester linkages on the periphery of the dendrimer.

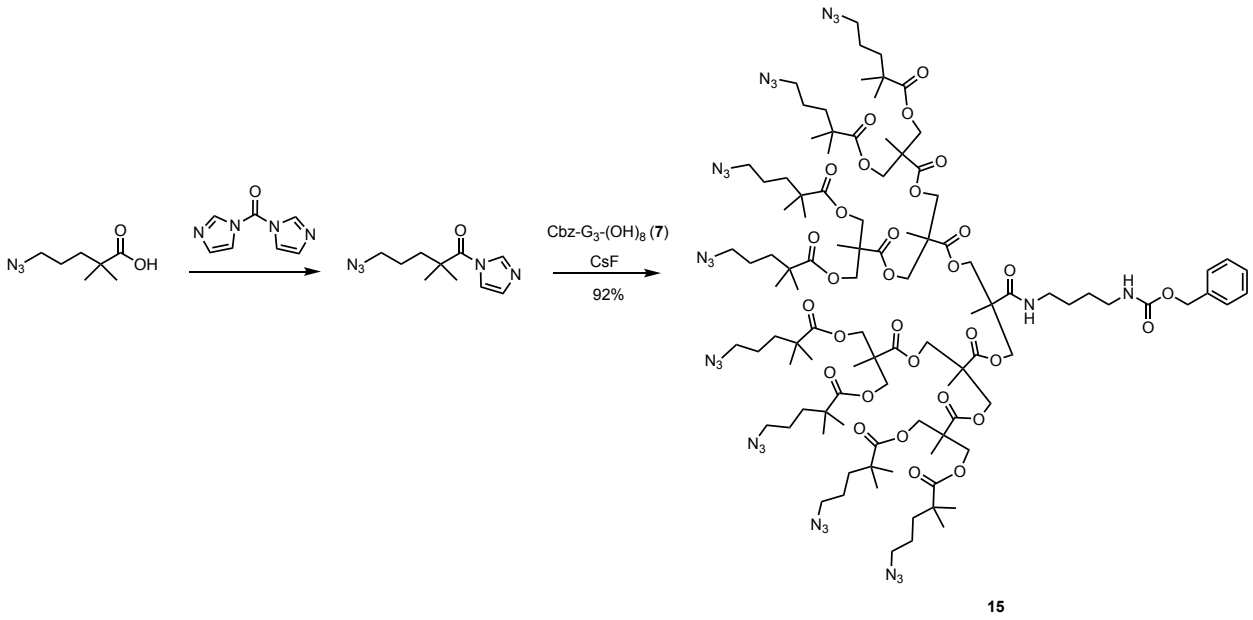


Scheme 2.3. Synthetic approach to prepare a neopentyl-azide functionalized linker.

The first step in preparing this linker involved deprotonation of ethyl-isobutyrate using a strong base, LDA. The resulting enolate could then attack 3-chloro-1-bromopropane in an S_N2 reaction, substituting the bromine group to afford **12**. The terminal chlorine could then be substituted with an azide group in another S_N2 reaction with sodium azide to give **13**. Finally, removal of the ethoxide group was accomplished using a saponification reaction with KOH to yield carboxylic acid **14**.

These linkers were then appended onto the periphery of the dendrimers to afford reactive handles which can later undergo PEGylation using SPAAC. The FPE was the first approach we used, which was first reported by Malkoch as an effective method to prepare bis-MPA dendrimers.[97] This method can also lend itself to functionalizing bis-MPA dendrimers using other carboxylic acid functionalized monomers. As illustrated in Scheme 2.4, this reaction first involves

the synthesis of an activated acyl imidazole which is done using CDI (4 eq./OH) and **14** (4 eq./OH). Once this activated species has been prepared, Cbz-G3-$(OH)_8$ was added along with catalytic CsF (1 eq./OH) in a 1 pot synthesis.

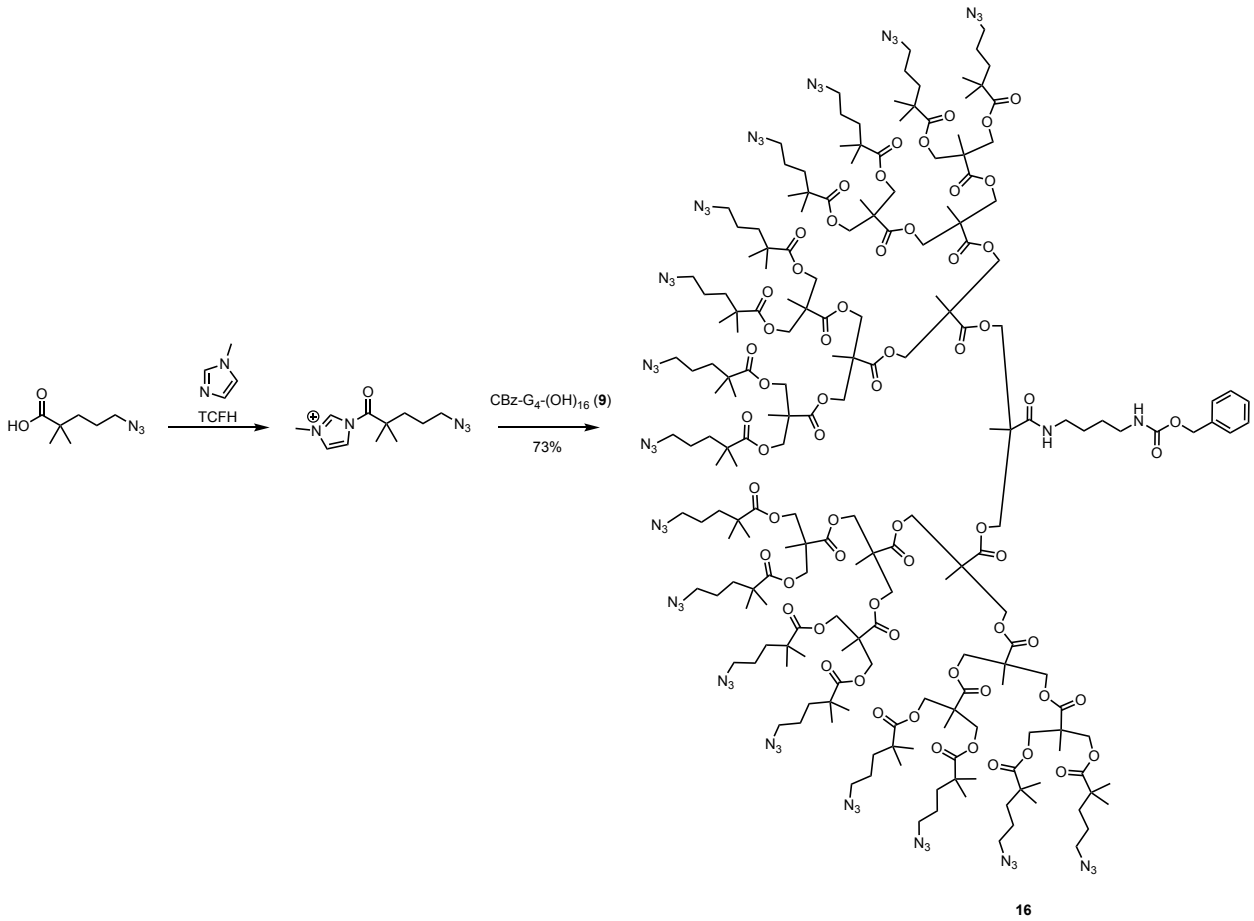


Scheme 2.4. Synthesis of Cbz-G3-$(N_3)_8$ using the FPE.

This reaction was initially unsuccessful as full conversion to the product was unattainable. Despite adding copious amounts of activated acyl imidazole, this reaction would not push toward completion. We hypothesized that a likely reason for this failure was due to the hydrolysis of both CDI and the activated imidazole in solution. To circumvent this, dry solvent was used and the reaction was performed under inert conditions. Although this modification had helped to obtain further conversion, it was still not entirely successful. Since the reaction vessel had to be continually opened in order to add more reagents, and/or take aliquots to monitor the reaction, the possibility of moisture entering the reaction vessel was unavoidable. Another reason why we believed this reaction to be unsuccessful was because of the scale on which it was performed. Since this reaction was done on very small scale, residual moisture in the reaction vessel would have a drastic effect. For example, one small scale reaction produced ~0.08 mmol of activated acyl

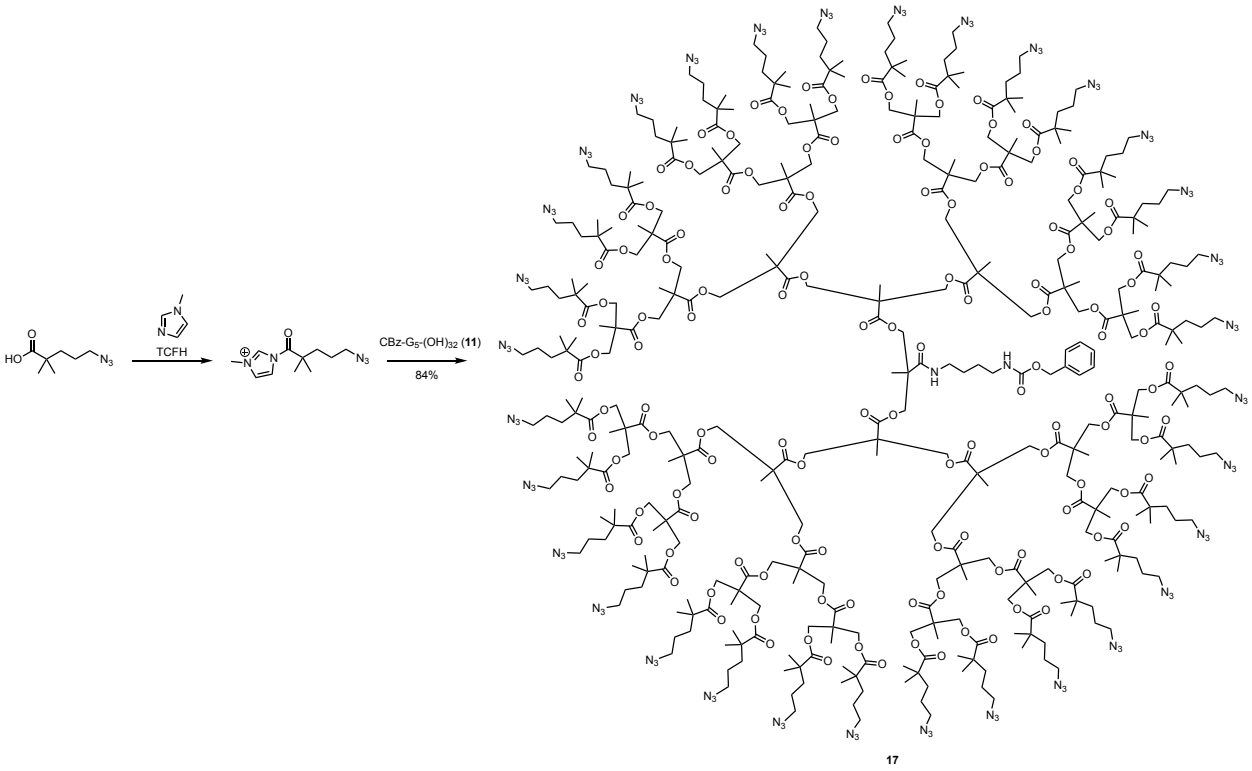
imidazole. It would therefore only take a few microliters of water to fully hydrolyze all of the activated acyl imidazole. To fix this issue, we scaled up the reaction by a factor of 50 and instead prepared ~4 mmol of activated acyl imidazole. This way it would take approximately one hundred microliters of water to fully hydrolyze the activated acyl imidazole, which would be far less likely when taking proper care to ensure inert reaction conditions. After taking these considerations into place, this reaction was completed successfully with the third generation dendrimer in a 92% yield. However, the reaction took just over 3 days to complete which is not ideal. For this reason, we sought out another method for functionalization of the G4 dendrimer, this time using a more reactive acyl transfer agent, an acyl imidazolium.

The inspiration for this method came from a paper published by Ye and co-workers.[98] In their work, they used NMI as an acyl transfer agent, and TCFH as an activating agent for a variety of amide couplings with poorly nucleophilic amines. As outlined in Scheme 2.5, we extended the chemistry they used for amide couplings to instead prepare esters using the dendrimers and **14**.



Scheme 2.5. Synthesis of Cbz-G4-$(N_3)_{16}$ using an acyl imidazolium.

Initial concerns about the hydrolysis of the acyl imidazolium were supressed by using dry solvent and inert reaction conditions as used previously in the FPE. The activated imidazolium was first prepared through reaction between **14** (2 eq./OH), NMI (3.5 eq./**14**), and TCFH (2 eq./OH). Next, in a one-pot synthesis the dendrimer was added and full conversion to the product was observed in just 30 minutes which was an exciting result as the total reaction time decreased by over 100-fold compared to the FPE. Due to the success of this method, it was again used to prepare the G5 azide periphery dendrimer as shown below in Scheme 2.6. Conversion to the desired product was achieved in under an hour, in 84% yield.



Scheme 2.6. Synthesis of Cbz-G5-$(N_3)_{32}$ using an acyl imidazolium.

To ensure that each of the product dendrimers was fully functionalized and monodisperse, MALDI-MS spectra were obtained for each one as shown in Figure 2.1. In addition to the expected m/z ratio being observed, a single distinguishable peak for each dendrimer indicates they are indeed monodisperse and no partially functionalized material is present.

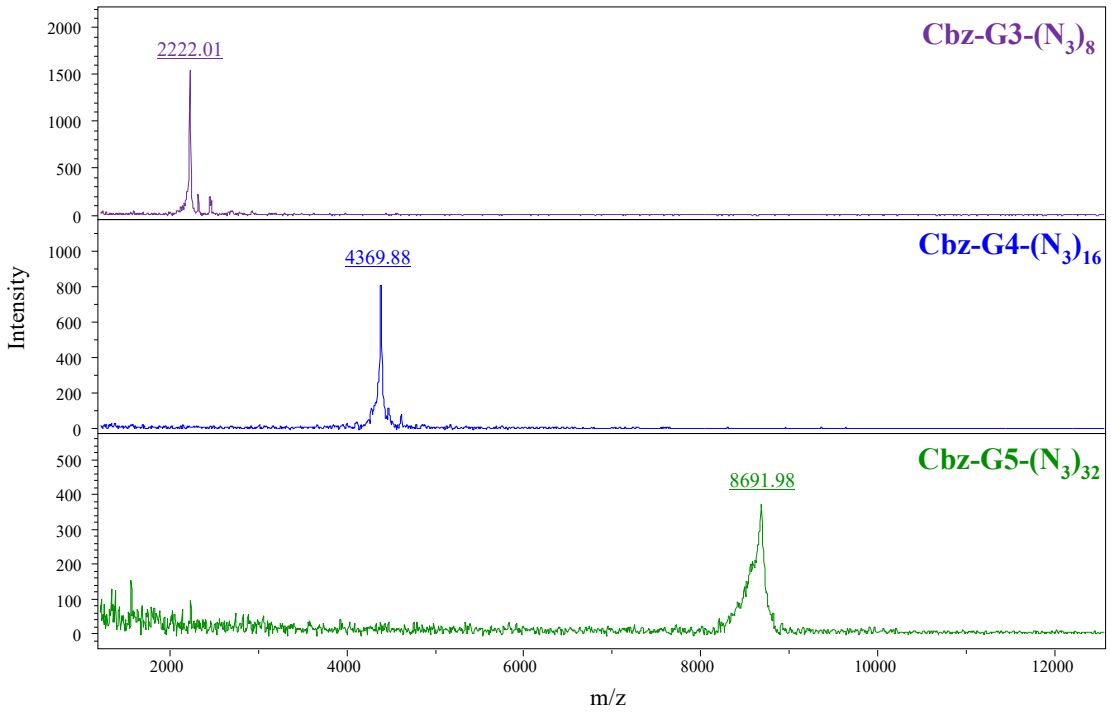
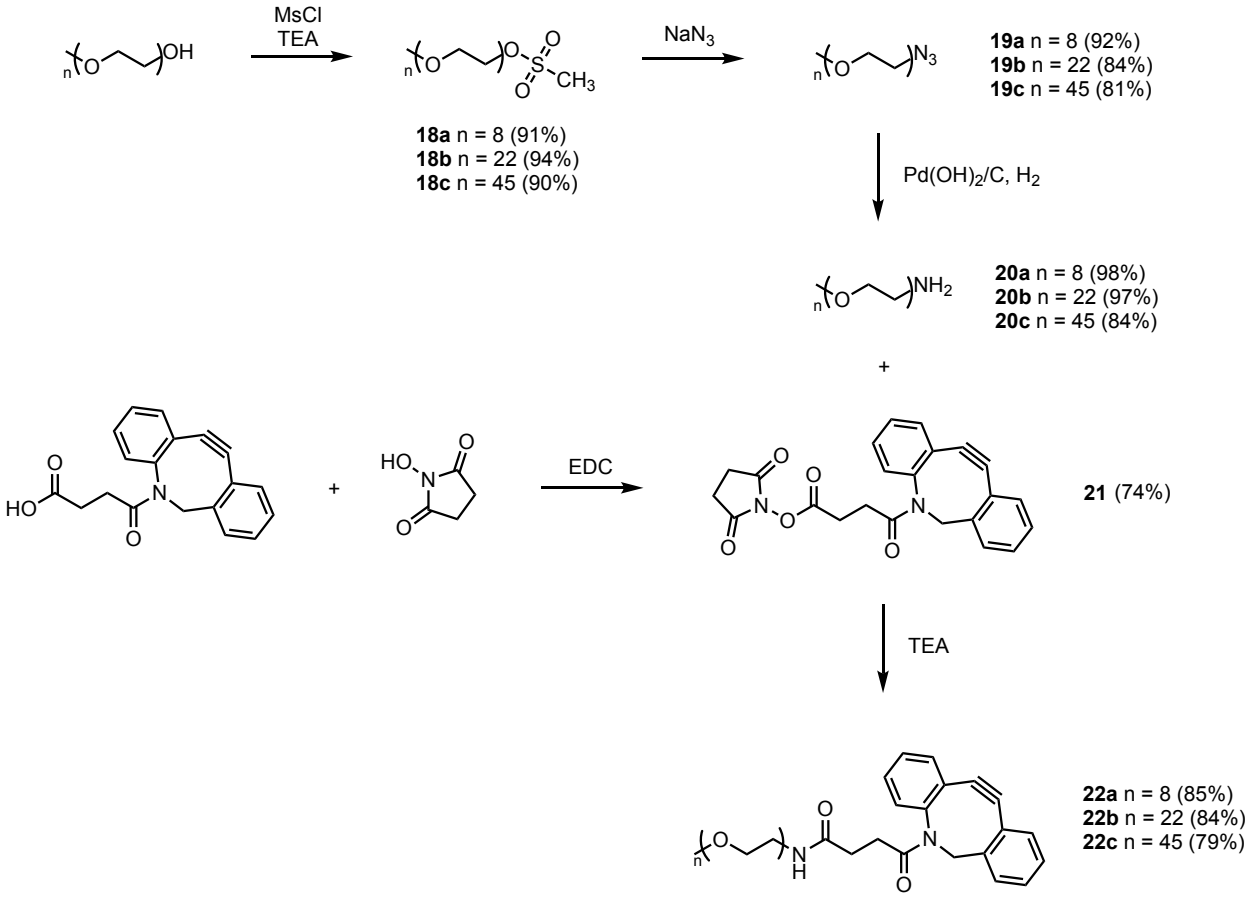


Figure 2.1. MALDI-MS spectra of Cbz-G3-$(N_3)_8$, Cbz-G4-$(N_3)_{16}$, and Cbz-G5-$(N_3)_{32}$, all illustrating full conversion to monodisperse product.

2.3 Synthesis of DBCO Functionalized PEG Chains

We set out to prepare amidated DBCO functionalized PEG chains as they are less prone to hydrolysis relative to those made with ester linkages, despite taking more steps to prepare. PEG chains that contained a protective methoxy group on one end were selected to ensure that only the one end of the PEG chain would be functionalized. This would avoid clicking of multiple azides with the same PEG chain. As illustrated in Scheme 2.7 the first step in this synthesis involved reaction between hydroxy terminated PEG, methanesulfonyl chloride and TEA. This formed PEG mesylate and made the hydroxyl group into a better leaving group for the next step which was an S_N2 reaction using sodium azide to yield azide functionalized PEG. The next step was a reduction of the azide group to afford a free amine. This was achieved through catalytic hydrogenation and FTIR was used to monitor this reaction through disappearance of the azide peak which absorbs IR

radiation at approximately 2100 cm^{-1}. Although this amine could react directly with DBCO-COOH, we chose to activate the DBCO with NHS to make it more reactive. Upon formation of DBCO-NHS, reaction with the amine functionalized PEG was completed to afford the amidated PEG-DBCO. This was completed for PEG-350, 1000, and 2000 (where n = 8, 22, and 45, respectively).

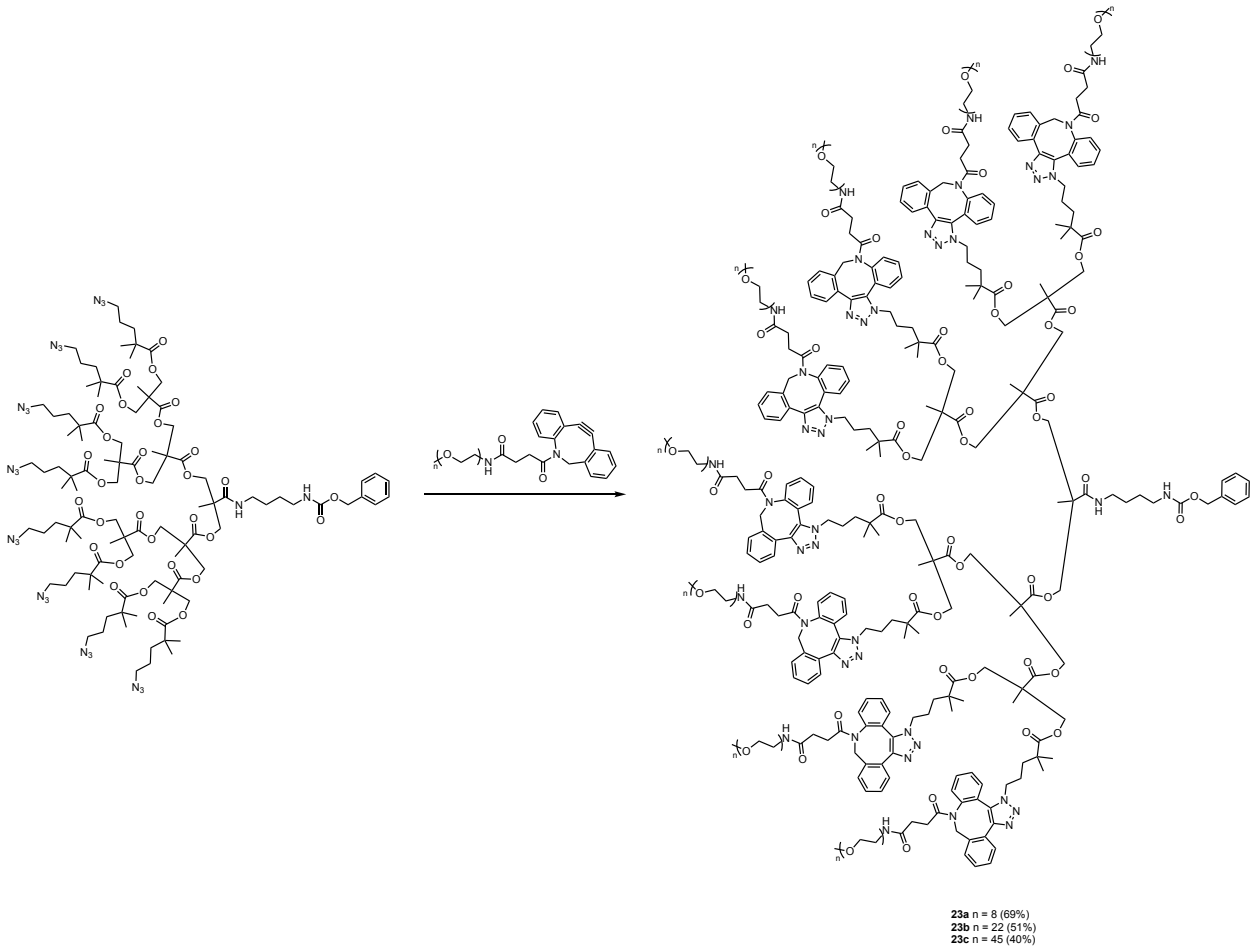


Scheme 2.7. Synthesis of amidated DBCO functionalized PEG chains.

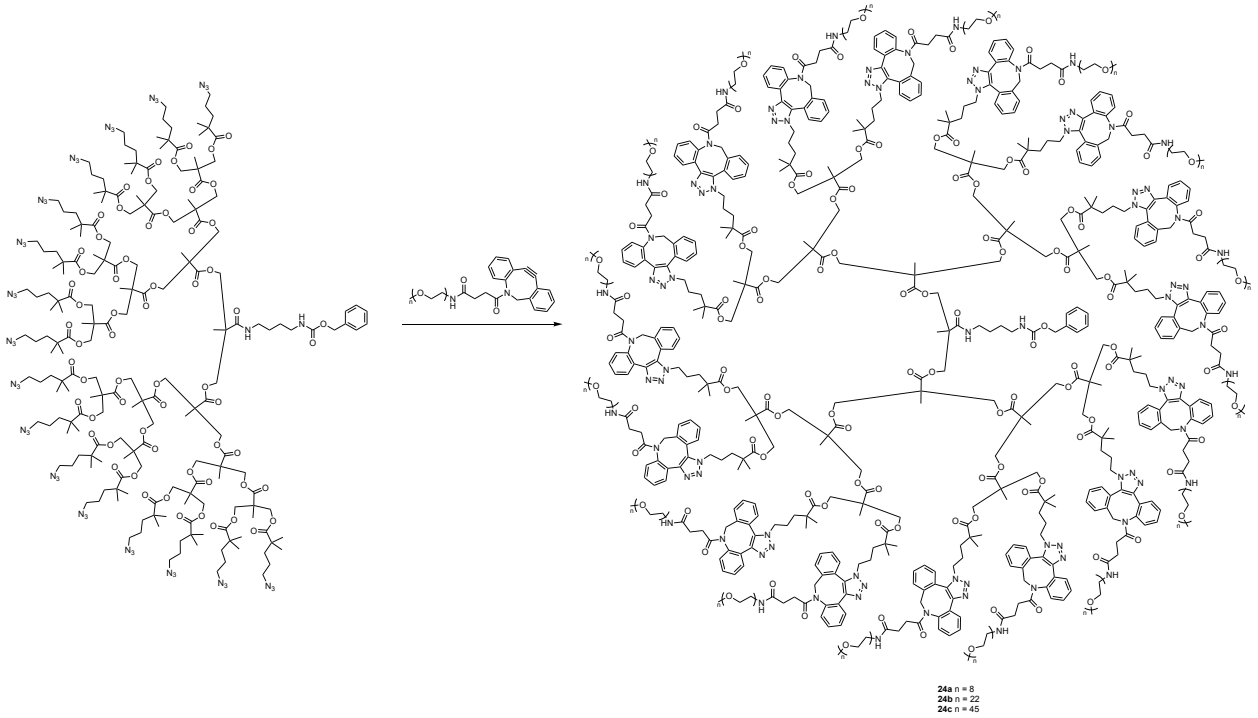
2.4 Dendrimer PEGylation Using SPAAC Chemistry

Equipped with azide functionalized dendrimers and DBCO functionalized PEG chains, dendrimer PEGylation was completed. As illustrated in Schemes 2.8, 2.9, and 2.10, PEGylation was achieved using the SPAAC reaction between the three azide periphery dendrimers prepared

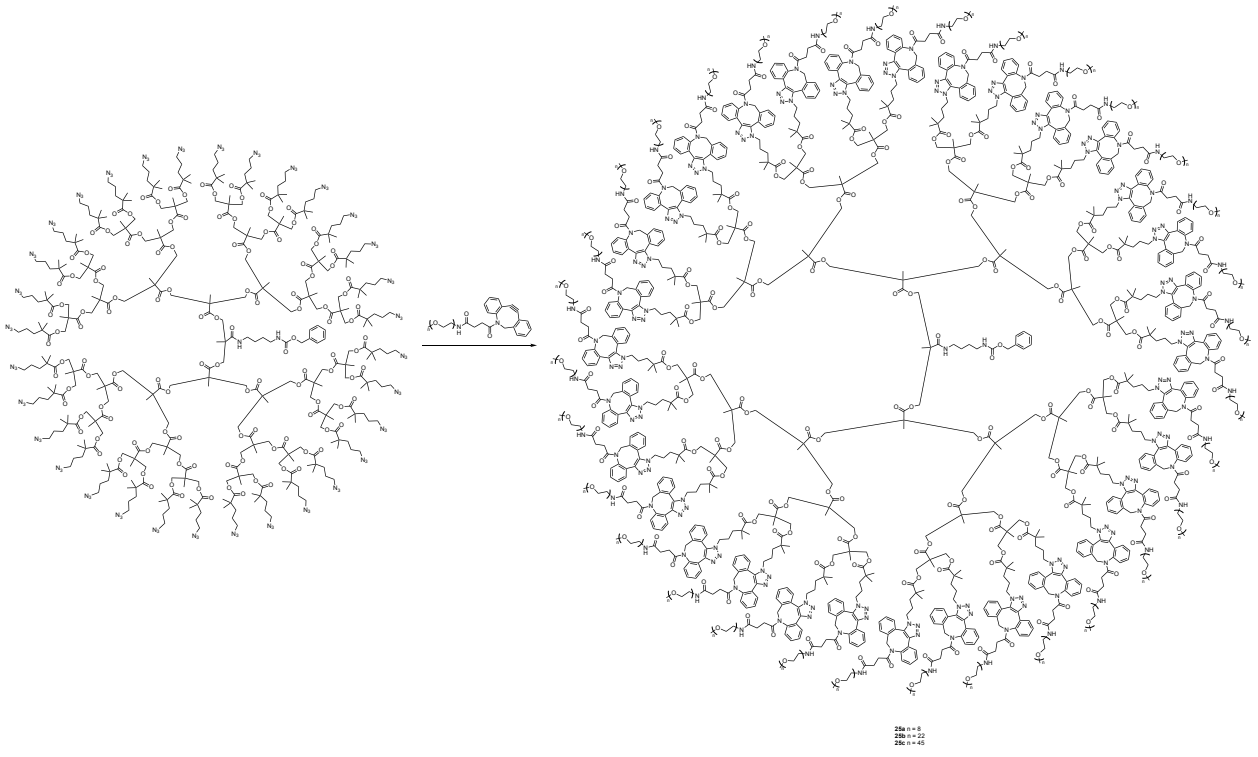
in section 2.2 and the three different molecular weight DBCO functionalized PEG chains synthesized in section 2.3. This afforded a library of 9 dendrimers each varying in generation and PEG chain molecular weight grafted to the periphery.



Scheme 2.8. Synthesis of Cbz-G3-$(mPEG)_8$ derivatives.



Scheme 2.9. Synthesis of Cbz-G4-$(mPEG)_{16}$ derivatives.



Scheme 2.10. Synthesis of Cbz-G5-$(mPEG)_{32}$ derivatives.

This reaction was carried out with ease and only took 1.5 eq. of DBCO-PEG per azide, and 20 minutes of stirring to fully PEGylate each azide functionalized dendrimer. FTIR was used to monitor the progression of these reactions and sample spectra are shown below in Figure 2.2. The black spectrum is the starting azide functionalized dendrimer and the blue spectrum is the reaction mixture after 20 minutes. Full disappearance of the azide signal at ~2100 cm^{-1} indicated that the reaction was complete as all azide groups had been clicked with DBCO moieties. Purification of this reaction to eliminate the excess DBCO functionalized PEG chains was also made easy through the use of dialysis, which was carried out overnight.

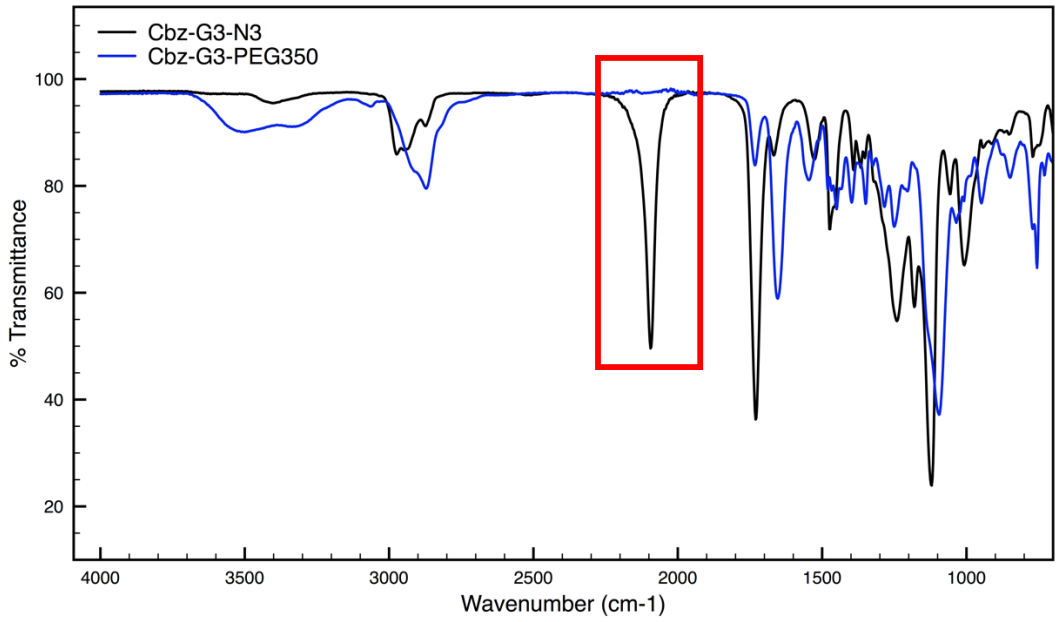


Figure 2.2. Sample FTIR spectrum illustrating dendrimer PEGylation using the SPAAC reaction. Red box highlights appearance/disappearance of azide signal.

To further prove that the azide functionalized dendrimers were fully PEGylated with each size of PEG chain, ^{1}H NMR spectroscopy was used to observe the change in chemical shift of key protons on the DBCO-PEG chain before, and after dendrimer PEGylation. Figure 2.3a shows the ^{1}H NMR spectrum of a sample DBCO functionalized PEG chain. The methylene protons on the

cyclooctyne ring can be observed as a doublet at 5.17 ppm, and another doublet at ~3.7 ppm, however, the latter doublet is hidden underneath the PEG signal and is not shown. Figures 2.3b-d show sample ^{1}H NMR spectra of fully PEGylated G3, G4, and G5 dendrimers, respectively. We consistently observe that upon clicking with the azide functionalized dendrimers, the doublet at 5.17 ppm shifts to 6.0 ppm. Figure 2.3e also illustrates this diagrammatically. This chemical shift post-click is thought to be due to further deshielding of the protons from the formation of a local aromatic triazole ring. The new signal now appears as a doublet of doublets due to the production of two isomers, which are typically produced in roughly equal amounts. The integration of this signal now becomes 8H for the G3 PEGylated dendrimers (Figure 2.3b) as 8 PEG chains have been clicked onto the periphery. Similarly, this value is 16H for the G4 dendrimers (Figure 2.3c), and 32H for the G5 dendrimers (Figure 2.3d). We also observe the expected integrations for amide protons that are present on the DBCO-PEG chains. Prior to PEGylation these protons can be been seen as a broad singlet at ~6.4 ppm. Post-click these protons have relatively similar chemical shifts, but the new integration values are representative of the number of DBCO-PEG chains that were clicked onto the periphery. As illustrated below, these peaks all integrate as expected to 8H for the G3 derivatives, 16H for the G4 derivatives, and 32H for the G5 derivatives.

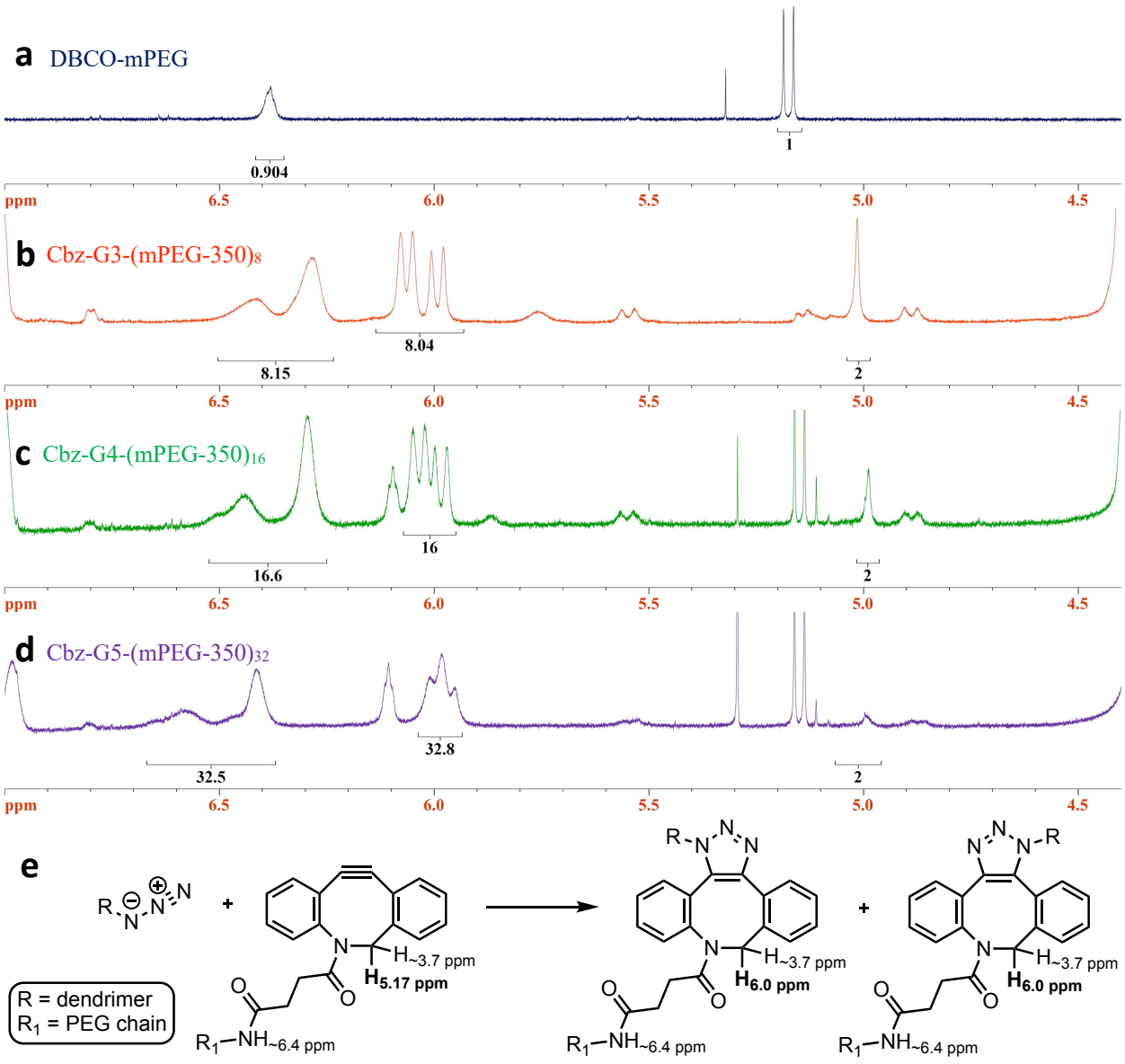
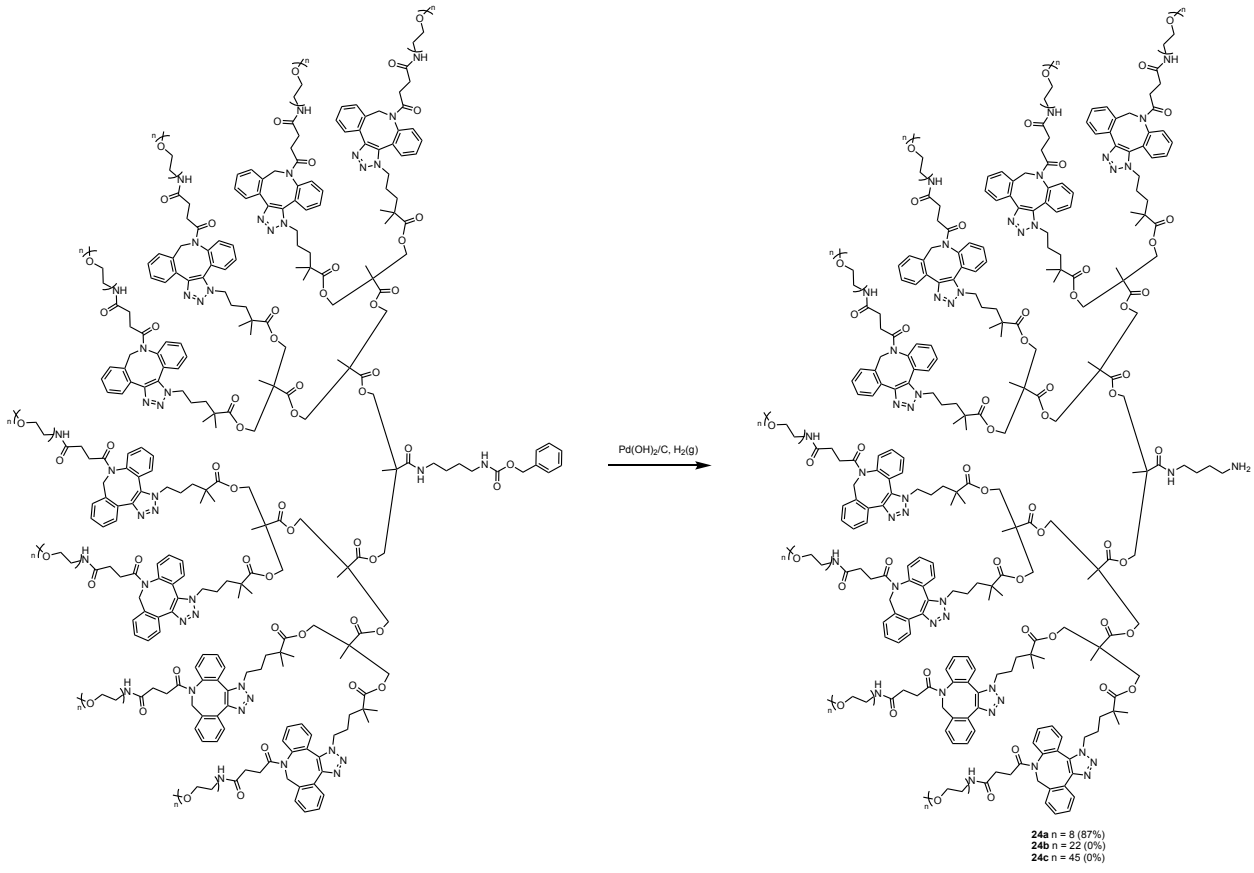


Figure 2.3. [1]HNMR spectra of: (a) a sample DBCO functionalized PEG chain, pre-click, (b) a G3 PEGylated dendrimer, (c) a G4 PEGylated dendrimer, (d) a G5 PEGylated dendrimer, all post click, with critical integral values illustrating complete PEGylation. Samples are referenced against the Cbz methylene at the dendrimer core (δ 5.0 ppm, 2H). Note: (c) and (d) represent crude reaction mixtures that still contain excess DBCO-PEG. (e) SPAAC reaction illustrating critical protons and their changes in chemical shift post-click.

The integration values from both the cyclooctyne and amide protons observed in ^{1}H NMR spectroscopy corroborate the earlier findings that were obtained through FTIR in that all of the azide periphery dendrimers have been fully functionalized with each size of PEG chain.

2.5 Core Deprotection

Prior to modifying the core with a DBCO moiety for future conjugation to the model enzyme, the core must first be deprotected. The most common way in the literature of removing a Cbz group is through hydrogenolysis using a $Pd(OH)_2/C$ catalyst with $H_{2(g)}$ as shown in Scheme 2.11. This method of deprotection has been used before in our group for the deprotection of bis-MPA dendrimers and has been done so with ease. However, this has not yet been done with PEGylated dendrimers.



Scheme 2.11. Deprotection of Cbz core through hydrogenolysis.

^{1}H NMR was used to monitor all core deprotection reactions in this section as the methylene protons in the Cbz group are chemically unique from all other protons in the molecule and can be easily distinguished at ~5.0 ppm. Upon removal of the Cbz group, this peak should disappear, however, this only occurred for the Cbz-G3-(PEG-350)$_8$ derivative and not for the other PEGylated G3 derivatives. As illustrated below in Figure 2.4, the Cbz protons in the starting material of Cbz-G3-(PEG-1000)$_8$ (bottom spectrum) are still present in the reaction mixture (top spectrum, t = 72 h) indicating that the reaction was not successful. A similar result was also observed for the Cbz-G3-(PEG-2000)$_8$ analog.

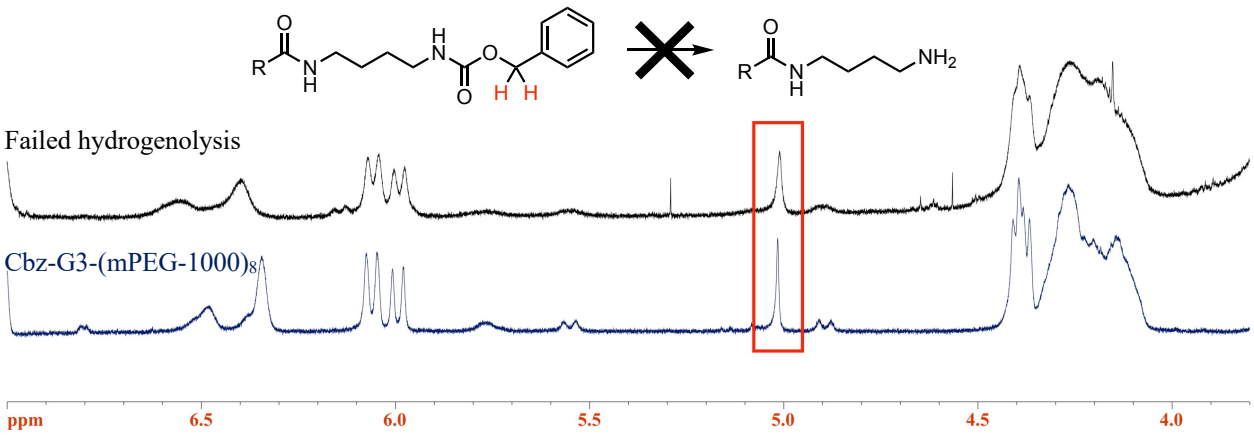


Figure 2.4. ^{1}H NMR spectra showing Cbz-G3-(PEG-1000)$_8$ (blue) and a sample reaction mixture (black) after 72 h of reaction time. Red box highlights presence of Cbz methylene peak (red protons in shown starting material) at ~ 5.0 ppm in both materials indicating no Cbz removal.

It was rather surprising that this reaction was unable to deprotect the cores of the PEG-1000 and PEG-2000 derivatives. Our group has been able to deprotect similar core dendrimers of higher generations, which had an even denser dendritic backbone than the one herein.[93] We hypothesize that there could be some intermolecular and/or intramolecular PEG entanglement which render the core inaccessible. With the use of the heterogeneous catalyst like Pd(OH)$_2$/C which acts as a surface onto which the core of the dendrimer needs to directly bind, PEG

entanglement would make it very difficult for this to happen, and for the reaction to occur. In an attempt to solve this issue, the amount of catalyst used, the temperature, and reaction time were all increased. Despite these changes, the reaction was still unsuccessful.

We changed our focus to a different catalyst, $Pd(PPh_3)_4$, which has also been reported for Cbz removal.[99] Unfortunately, this catalyst was again unable to remove the Cbz group of the dendrimers, likely due to its extreme air sensitivity. Despite taking care to ensure an inert reaction environment, the catalyst likely decomposed before any reaction could take place.

Final attempts to get this reaction to proceed involved using different salts that provide a fluoride or bromide ion, which have been used in the literature for Cbz group removal.[100] TBAF, TBABr, and TEAF were each attempted in different molar equivalents while altering other parameters such as the reaction time and temperature. All three of these were again unable to achieve the desired result, so we decided to go back and test the ability of these compounds to remove the Cbz group of a smaller non-PEGylated dendrimer. While TBABr and TEAF did not yield any Cbz removal of Cbz-G2-$(OH)_4$, TBAF consumed Cbz-G2-$(OH)_4$ as observed by mass spectrometry. However, it did not yield any of the expected product. Since this result was rather interesting, TBAF was used to attempt to remove the core of Cbz-G3-$(PEG\text{-}1000)_8$. The material obtained was then reacted with DBCO-NHS and TEA. Our hypothesis was that in the event that the Cbz group was actually removed, any free amine would react directly with DBCO-NHS and yield the desired DBCO core dendrimer. However, upon reaction with DBCO-NHS and TEA overnight, there was no clear indication that any DBCO had been coupled onto the core of the dendrimer after purification. These results showed that either no Cbz cleavage occurred to begin with, or an unknown side reaction occurred yielding something that was not a free amine. Table 2.1 outlines the various reagents and reaction conditions attempted to remove the Cbz core of Cbz-

G3-(PEG-1000)$_8$ and Cbz-G3-(PEG-2000)$_8$. Similar to our earlier hypothesis with the hydrogenolysis reactions, we suspect that either intermolecular and intramolecular PEG entanglement render the core completely inaccessible for core modifications, or, an unknown side reaction is occurring which does not produce a free amine.

Table 2.1. Attempts to remove Cbz core of Cbz-G3-(PEG-1000)$_8$ and Cbz-G3-(PEG-2000)$_8$.

Trial	Reagents	Solvent	Time	Temperature	Yield[1]
1a	$Pd(OH)_2/C$ (5%) & $H_{2(g)}$	1:1 DCM:MeOH	24 h, 48 h	25°C	Negligible
1b	$Pd(OH)_2/C$ (10%) & $H_{2(g)}$	1:1 DCM:MeOH	24 h, 48 h	25°C	Negligible
1c	$Pd(OH)_2/C$ (50%) & $H_{2(g)}$	1:1 DCM:MeOH	24 h, 48 h, 72 h	35°C	Negligible
2	$Pd(PPh_3)_4$ (10%), K_2CO_3 (5 eq.)	MeOH	4 h, 24 h	25°C	Negligible
3a	TBABr (1.2 eq.)	Dry THF	5 h	75°C	Negligible
3b	TBABr (5 eq.)	Dry THF	24 h	75°C	Negligible
4a	TEAF (1.2 eq.)	Dry THF	5 h	75°C	Negligible
4b	TEAF (5 eq.)	Dry THF	24 h	75°C	Negligible
5a	TBAF (5 eq.)	Dry THF	24 h	75°C	Negligible
5b	TBAF (10 eq.)	Dry THF	24 h	75°C	Negligible

[1]Based on NMR data (similar to Figure 2.4), the methylene protons were not observed to undergo any significant changes.

Although the literature has several other possibilities for Cbz group removal, we were limited by either the availability of reagents, or the incompatibility of the reaction conditions with the dendrimers. For example, some reported methods involve reaction conditions that require highly concentrated basic conditions that needed to react for several hours under high temperatures.[101,102] While these methods might have worked for this series of compounds, the conditions would likely hydrolyze the ester linkages in the dendrimer backbone and/or the amide linkages present in the PEG chains. As a result, further attempts to remove the cores of Cbz-G3-(PEG-1000)$_8$ and Cbz-G3-(PEG-2000)$_8$ were not attempted, nor were attempts made to remove the

cores of the G4 and G5 analogs as the PEG grafting density on these was even more substantial. Instead, an entirely new approach was developed and will be the focus of the next chapter.

2.6 Conclusion

A divergent synthesis method was employed to prepare generation 1-5 bis-MPA dendrimers. The periphery of these dendrimers was then functionalized with a small molecule neo-pentyl azide linker which was prepared in a 3 step synthesis. The peripheries of the G3-G5 dendrimers were functionalized with this linker using one of two methods involving an activated acyl imidazole/imidazolium, the latter of which allowed for full functionalization in under an hour. Amidated DBCO functionalized PEG chains were then prepared in a four step synthesis and were easily clicked to the G3, G4, and G5 azide periphery dendrimers using SPAAC chemistry. Complete PEGylation of all dendrimers was achieved in just minutes. Deprotection of the Cbz core was achieved through hydrogenolysis, however only for the Cbz-G3-(PEG-350)$_8$ derivative. The cores of Cbz-G3-(PEG-1000)$_8$ and Cbz-G3-(PEG-2000)$_8$ were unable to be deprotected despite the use of many different reagents and reaction conditions explored. This was likely due to entanglement of the longer PEG chains which completely isolated the core. Attempts to deprotect the cores of the higher generation PEGylated dendrimers were not performed. Since the cores of these PEGylated dendrimers cannot be manipulated, this severely limits applications of the work presented in this chapter.

2.7 Experimental

General

All reagents and solvents were obtained from commercial sources and were used without further purification. DBCO derivatives were previously synthesized by Dr. Stuart McNelles. All anhydrous solvents were dried using 3 or 4 Å molecular sieves overnight prior to use. Flash

chromatography was performed using an IntelliFlash 280 system from Analogix and monitored using a variable wavelength detector. Columns were prepared using Sorbtech EZ Flash Column cartridges with 25 – 40 μm silica purchased from Silicycle. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Advance 600 MHz and 150 MHz spectrometers respectively. All chemical shifts are reported in ppm. FTIR measurements were taken on a Nicolet 6700 FTIR from Thermo Scientific. ESI-MS spectra were obtained using a Waters-Micromass Quattro Ultima Electrospray Ionization Mass Spectrometer, or an Agilent G1969 Time-of-Flight Mass Spectrometer. MALDI-MS measurements were taken on an UltrafleXtreme (Bruker Daltonics, MA, USA) in reflectron positive ion mode. Sample preparation is as follows: 1 mg of dithranol matrix was dissolved in 100 μL of THF and centrifuged for 1 min. Separately, an approximate 0.5 mg/mL solution of dendrimer was prepared in DCM. 10 μL of the dendrimer solution was mixed with 10 μL of the matrix solution. 1 μL of this solution was spotted onto the MALDI target plate and left to air dry until crystalized.

General Synthetic Procedures

General Procedure 1: Dendrimer Growth G2-G5 - *Modified from literature procedures*[103]

A round bottom flask was equipped with a magnetic stir bar and charged with 4 eq./OH of bis-MPA-acetonide (**1**), 2 eq./OH of EDC, and DCM. The mixture was stirred at room temperature for 30 min. Separately, 1 eq. of Cbz-Gx-$(OH)_y$, and 0.25 eq./OH of DMAP were dissolved in pyridine and were then added to the mixture followed by 2eq./OH of TEA. The reaction mixture was left stirring overnight at which point it was quenched with 1 mL of water and stirred for 1 hour. The crude mixture was diluted with 50 mL of DCM and then washed with 1M H_3PO_4 (3 x 50 mL), 10% Na_2CO_3 (3 x 50 mL), and brine (1 x 50 mL). The organic layer was dried with $MgSO_4$, filtered, then concentrated by rotary evaporation. The crude material was purified by flash

chromatography using a 40 g silica Sorbtech EZ Flash column with 10-60% acetone in hexanes (loaded in DCM) and monitored at 205 nm. Fractions containing product were combined, concentrated by rotary evaporation and dried under vacuum to afford the product as a clear viscous oil.

General Procedure 2: Dendrimer Deprotection G1-G5 - *Prepared according to literature procedures*[104]

A round bottom flask was equipped with a magnetic stir bar and charged with 1 eq. of Cbz-Gx-$(acet)_y$, MeOH, and Dowex® 50WX2 beads. This was left stirring at room temperature until completion. The mixture was vacuum filtered and the solution was concentrated by rotary evaporation, then dried under vacuum to afford the product as a viscous pale yellow oil.

General Procedure 3: Synthesis of mPEG-OMs - *Modified from literature procedures*[105,106]

A round bottom flask was equipped with a magnetic stir bar and charged with 1 eq. of mPEG-OH, DCM, and 4 eq. of TEA. The reaction mixture was cooled down to 0 °C after which 5 eq. of MsCl were added dropwise. The reaction vessel was then warmed to room temperature and stirred until complete at which point it was quenched with water and then stirred for an additional 15 min. The mixture was then washed with 10% Na_2CO_3 (3 x 60 mL) and 1M HCl (3 x 60 mL). The organic layers were dried with $MgSO_4$, filtered, and then concentrated under rotary evaporation. The product was then either directly dried under vacuum, or precipitated into diethyl ether, filtered, then dried under vacuum, depending on the PEG chain length.

General Procedure 4: Synthesis of mPEG-NH_2 - *Modified from literature procedures*[107]

A round bottom flask was equipped with a magnetic stir bar and charged with 1 eq. of mPEG-N_3, 1:1 MeOH:DCM, and 10% (by weight) of $Pd(OH)_2$/C. The reaction vessel was purged under vacuum and backfilled with hydrogen gas three times while stirring, and was then left stirring at

room temperature overnight under hydrogen gas. Disappearance of the azide peak in FTIR at ~ 2100 cm^{-1} was used to determine that the reaction was complete. The next day the reaction mixture was filtered using a 0.2 µm syringe filter and then either precipitated into diethyl ether, filtered, and dried under vacuum, or, concentrated by rotary evaporation and then dried under vacuum depending on the PEG chain length, to afford the product.

General Procedure 5: Synthesis of mPEG-DBCO - *Modified from literature procedures*[93]

A round bottom flask was equipped with a magnetic stir bar and charged with 1 eq. of mPEG-NH_2, DCM, 1.5 eq. of DBCO-NHS and 2 eq. of TEA. The reaction was left stirring at room temperature overnight. The next day the reaction was either concentrated by rotary evaporation, precipitated into diethyl ether, and then dried under vacuum, or, directly purified by flash chromatography first using a 40 g neutral alumina column with 20% MeOH in DCM, followed by a 120 g silica Sorbtech EZ Flash column with 0-5% MeOH in DCM and monitored at 254 nm, depending on the PEG chain length, to afford the product.

General Procedure 6: Dendrimer PEGylation - *Modified from literature procedures*[88]

A round bottom flask was equipped with a magnetic stir bar and charged with 1 eq. of Cbz-Gx-$(N_3)_y$, DCM, and 1.5 eq./N_3 of mPEG-DBCO. The reaction was left stirring at room temperature for 20 minutes and disappearance of the azide peak in FTIR at ~ 2100 cm^{-1} was used to determine that the reaction was complete. The solvent was removed by rotary evaporation and the mixture was dissolved in ~2 mL of deionized water and purified by dialysis overnight using either 3.5 kDa, 50 kDa, or 100 kDa MWCO dialysis tubing to remove excess mPEG-DBCO. Dialysis water was changed three times over the span of a 24 h period. The solution was then lyophilized overnight to afford the product.

Synthetic Procedures

Bis-MPA-acetonide (1) - *Prepared according to literature procedures*[104]

A round bottom flask was equipped with a magnetic stir bar and charged with 2,2-bis(hydroxymethyl)propionic acid (23.5 g, 175 mmol), 2,2-dimethoxypropane (32 mL, 263 mmol), p-toluenesulfonic acid (1.67 g, 8.76 mmol) and acetone (125 mL). This was left stirring at room temperature for 4 hours. The mixture was then quenched with ammonia in water (2.35 mL) and stirred for 5 minutes. The solvent was evaporated and the crude material was dissolved in 590 mL of DCM and washed with water (3 x 50 mL). The organic layer was dried with $MgSO_4$, filtered, and then concentrated by rotary evaporation and dried under vacuum to afford the product as a white powder. (26.352 g, 86%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 4.16 (d, *J* = 11.95 Hz, 2H), 3.66 (d, *J* = 11.95 Hz, 2H), 1.43 (s, 3H), 1.40 (s, 3H).

HRMS (ESI^+) *m/z* calc'd for $C_8H_{14}O_4$ $[M+Na]^+$ = 197.0784, found $[M+Na]^+$ 197.0767.

Cbz-G1-(acet)$_1$ (2)

A round bottom flask was equipped with a magnetic stir bar and charged with bis-MPA-acetonide (1.32 g, 7.61 mmol), EDC (729 mg, 3.80 mmol) and DCM (6.3 mL). This was left stirring at room temperature for 30 min at which point *N*-Z-1,4-butanediamine hydrochloride (820 mg, 3.17 mmol) and TEA (1.6 mL, 11.10 mmol) were added. The mixture was left stirring overnight. The following day the reaction mixture was diluted with 50 mL of DCM and washed with 1M H_3PO_4 (3 x 50 mL), 10% Na_2CO_3 (3 x 50 mL), and brine (1 x 50 mL). The organic layer was dried with $MgSO_4$, filtered, and then concentrated by rotary evaporation. The crude material was purified by flash chromatography using a 40 g silica Sorbtech EZ Flash column with 10-60% acetone in hexanes (loaded in DCM) and monitored at 205 nm. Fractions containing product were combined,

concentrated by rotary evaporation and dried under vacuum to afford the product as a clear oil. (1.151 g, 96%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.33-7.28 (m, 5H), 7.09 (s, 1H), 5.06 (s, 2H), 4.82 (s, 1H) 3.87 (d, J = 12.16 Hz, 2H), 3.73 (d, J = 12.28 Hz, 2H), 3.30-3.20 (m, 4H), 1.58-1.54 (m, 4H), 1.45 (s, 3H), 1.39 (s, 3H), 0.97 (s, 3H).

HRMS (ESI$^+$) *m/z* calc'd for $C_{20}H_{30}N_2O_5$ $[M+Na]^+$ = 401.2047, found $[M+Na]^+$ 401.2051.

Cbz-G1-(OH)$_2$ (3)

Using general procedure 2, Cbz-G1-(acet)$_1$ (745 mg, 1.97 mmol) was dissolved in 110 mL of MeOH followed by the addition of Dowex® (1.12 g) and stirred for 3 hours. (666 mg, 99%).

^{1}H NMR (600 MHz; MeOD): δ 7.34-7.27 (m, 5H), 5.09 (s, 2H), 3.65 (d, J = 10.94 Hz, 2H), 3.60 (d, J = 10.94 Hz, 2H), 3.22-3.12 (m, 4H), 1.53-1.49 (m, 4H), 1.09 (s, 3H).

HRMS (ESI$^+$) *m/z* calc'd for $C_{17}H_{26}N_2O_5$ $[M+Na]^+$ = 361.1734, found $[M+Na]^+$ 361.1736.

Cbz-G2-(acet)$_2$ (4)

Using general procedure 1, bis-MPA-acetonide (3.292 g, 18.9 mmol) and EDC (1.813 g, 9.46 mmol) were dissolved in 5.6 mL of DCM followed by addition of Cbz-G1-(OH)$_2$ (**3)** (800 mg, 2.36 mmol), DMAP (144 mg, 1.18 mmol) and TEA (1.3 mL, 9.46 mmol), in 3.8 mL of pyridine. (1.445 g, 94%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.35-7.28 (m, 5H), 6.50 (s, 1H), 5.07 (s, 2H), 4.93 (s, 1H), 4.33 (d, J = 11.25 Hz, 2H), 4.26 (d, J = 11.26 Hz, 2H), 4.14 (d, J = 11.90 Hz, 4H), 3.63-3.61 (m, 4H), 3.26-3.15 (m, 4H), 1.52-1.47 (m, 4H), 1.39 (s, 6H), 1.32 (s, 6H), 1.23 (s, 3H), 1.07 (s, 6H).

HRMS (ESI$^+$) *m/z* calc'd for $C_{33}H_{50}N_2O_{11}$ $[M+Na]^+$ = 673.3313, found $[M+Na]^+$ 361.3316.

Cbz-G2-(OH)$_4$ (5)

Using general procedure 2, Cbz-G2-(acet)$_2$ (1.654 g, 2.54 mmol) was dissolved in 145 mL of MeOH followed by the addition of Dowex® (6.25 g) and stirred for 3 hours. (1.448 g, 99%).

^{1}H NMR (600 MHz; MeOD): δ 7.37-7.30 (m, 5H), 5.06 (s, 2H), 4.28-4.22 (m, 4H), 3.68 (d, *J* = 10.84 Hz, 2H), 3.59 (d, *J* = 10.87 Hz, 4H), 3.23-3.11 (m, 4H), 1.56-1.48 (m, 4H), 1.27 (s, 3H), 1.15 (s, 6H).

HRMS (ESI$^+$) *m/z* calc'd for $C_{27}H_{42}N_2O_{11}$ $[M+Na]^+$ = 593.2687, found $[M+Na]^+$ 593.2693.

Cbz-G3-(acet)$_4$ (6)

Using general procedure 1, bis-MPA-acetonide (5.662 g, 32.5 mmol) and EDC (3.118 g, 16.3 mmol) were dissolved in 7 mL of DCM, followed by addition of Cbz-G2-(OH)$_4$ (**5)** (1.16 g, 2.03 mmol), DMAP (248 mg, 2.03 mmol) and TEA (2.3 mL, 16.3 mmol), in 4.7 mL of pyridine. (2.306 g, 95%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.33-7.27 (m, 5H), 6.37 (s, 1H), 5.39 (s, 1H), 5.06 (s, 2H), 4.33-4.17 (m, 12H), 4.13-4.09 (m, 8H), 3.61-3.56 (m, 8H), 3.28-3.17 (m, 4H), 1.56-1.50 (m, 4H), 1.37 (s, 12H), 1.30 (s, 12H), 1.23-1.21 (m, 9H), 1.08 (s, 12H).

HRMS (ESI$^+$) *m/z* calc'd for $C_{59}H_{90}N_2O_{23}$ $[M+H]^+$ = 1195.6007, found $[M+H]^+$ 1195.6015.

Cbz-G3-(OH)$_8$ (7)

Using general procedure 2, Cbz-G3-(acet)$_4$ (870 mg, 0.73 mmol) was dissolved in 330 mL of MeOH followed by the addition of Dowex® (8.7 g) and stirred 3 hours. (745 mg, 98%).

^{1}H NMR (600 MHz; MeOD): δ 7.38-7.27 (m, 5H), 5.07 (s, 2H), 4.31-4.21 (m, 12H), 3.68-3.66 (m, 8H), 3.57-3.60 (m, 8H), 3.23-3.13 (m, 4H), 1.57-1.49 (m, 4H), 1.29-1.27 (m, 9H), 1.15 (s, 12H).

HRMS (ESI$^+$) *m/z* calc'd for $C_{47}H_{74}N_2O_{23}$ $[M+H]^+$ = 1035.4755, found $[M+H]^+$ 1035.4767.

Cbz-G4-(acet)$_8$ (8)

Using general procedure 1, bis-MPA-acetonide (3.773 g, 21.70 mmol) and EDC (2.077 g, 10.8 mmol) were dissolved in 7 mL of DCM, followed by addition of Cbz-G3-(OH)$_8$ (**7**) (701 mg, 0.68 mmol), DMAP (165 mg, 1.35 mmol) and TEA (1.5 mL, 10.8 mmol), in 4.6 mL of pyridine. (1.485 g, 96%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.33-7.26 (m, 5H), 6.48 (s, 1H), 5.36 (s, 1H), 5.05 (s, 2H), 4.31-4.20 (m, 28H), 4.13-4.09 (m, 16H), 3.61-3.57 (m, 16H), 3.27-3.15 (m, 4H), 1.55-1.51 (m, 4H), 1.38 (s, 24H), 1.32 (s, 24H), 1.27-1.23 (m, 21H), 1.11 (s, 24H).

MALDI-MS *m/z* calc'd for $C_{111}H_{170}N_2O_{47}$ $[M+Na]^+$ = 2306.0872, found $[M+Na]^+$ 2306.3891.

Cbz-G4-(OH)$_{16}$ (9)

Using general procedure 2, Cbz-G4-(acet)$_8$ (323 mg, 0.14 mmol) was dissolved in 180 mL of MeOH followed by the addition of Dowex® (6.1 g) and stirred for 5.5 hours. (271 mg, 97%).

^{1}H NMR (600 MHz; MeOD): δ 7.36-7.34 (m, 5H), 5.07 (s, 2H), 4.33-4.24 (m, 28H), 3.69-3.66 (m, 16H), 3.62-3.59 (m, 16H), 3.25-3.14 (m, 4H), 1.57-1.53 (m, 4H), 1.33-1.28 (m, 21H), 1.14 (s, 24H).

MALDI-MS *m/z* calc'd for $C_{87}H_{138}N_2O_{47}$ $[M+K]^+$ = 2001.8107, found $[M+Na]^+$ 2001.9017.

Cbz-G5-(acet)$_{16}$ (10)

Using general procedure 1, bis-MPA-acetonide (1.265 g, 7.27 mmol) and EDC (697 mg, 3.63 mmol) were dissolved in 1.7 mL of DCM, followed by addition of Cbz-G4-(OH)$_{16}$ (**9**) (223 mg, 0.11 mmol), DMAP (55 mg, 0.45 mmol), and TEA (0.5 mL, 3.63 mmol), in 1.2 mL of pyridine. (484 mg, 95%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.31-7.26 (m, 5H), 6.54 (s, 1H), 5.51 (s, 1H), 5.04 (s, 2H), 4.33-4.18 (m, 60H), 4.11-4.07 (m, 32H), 3.61-3.56 (m, 32H), 3.25-3.14 (m, 4H), 1.54-1.49 (m, 4H), 1.38 (s, 48H), 1.34 (s, 48H), 1.29-1.22 (m, 45H), 1.11 (s, 48H).

MALDI-MS *m/z* calc'd for $C_{215}H_{330}N_2O_{95}$ $[M+Na]^+$ = 4483.0951, found $[M+Na]^+$ 4482.6364.

Cbz-G5-(OH)$_{32}$ (11)

Using general procedure 2, Cbz-G5-(acet)$_{16}$ (440 mg, 0.099 mmol) was dissolved in 60 mL of MeOH followed by the addition of Dowex® (400 mg) and stirred for 6 hours. (371 mg, 99%).

^{1}H NMR (600 MHz; MeOD): δ 7.31-7.25 (m, 5H), 5.03 (s, 2H), 4.31-4.19 (m, 60H), 3.66-3.60 (m, 32H), 3.58-3.54 (m, 32H), 3.22-3.10 (m, 4H), 1.55-1.48 (m, 4H), 1.32-1.23 (m, 45H), 1.13 (s, 48H).

MALDI-MS *m/z* calc'd for $C_{167}H_{266}N_2O_{95}$ $[M+K]^+$ = 3858.5682, found $[M+K]^+$ 3871.5538.

Ethyl 5-chloro-2,2-dimethylpentanoate (12)

A 250 mL flame dried Schlenk flask under nitrogen was equipped with a magnetic stir bar and charged with diisopropylamine (7.24 mL, 51.7 mmol) added via syringe, and 120 mL of dry THF added via cannula. The solution was cooled to -78 ºC in a dry ice/acetone bath and was left to cool for 10 minutes while stirring. To this, a solution of 2.5 M n-butyllithium in hexanes (17.7 mL, 43.0 mmol) was added dropwise using a cannula and an addition funnel, which resulted in a solution of LDA. Separately, ethyl isobutyrate (5.0 g, 43.0 mmol) was dissolved in 25 mL of dry THF and cooled to -78 ºC in a dry ice/acetone bath. This was cannulated dropwise into the solution of LDA, and left stirring for 30 minutes at -78 ºC at which point 3-chloro-1-bromopropane (6.4 mL, 64.6 mmol) was added as a neat liquid via syringe. This was left to react for 30 minutes at -78 ºC, and then brought to room temperature and stirred for 3 hours. The reaction was then quenched with 50 mL of water, diluted with 300 mL of diethyl ether, washed with 1 M HCl (3 x 100 mL), brine (1

x 150 mL), dried with $MgSO_4$, filtered, and solvent was removed by rotary evaporation. The crude material was purified by flash chromatography using a 120 g silica Sorbtech EZ Flash column with 5 column volumes of 100% hexanes followed by 20 column volumes of a gradient containing 0-15% diethyl ether in hexanes. Fractions containing product were combined, concentrated by rotary evaporation and dried under vacuum to afford the product as a clear liquid. (3.511 g, 42%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 4.12 (q, *J* = 7.12 Hz, 2H), 3.51 (t, *J* = 6.45 Hz, 2H), 1.74-1.70 (m, 2H), 1.67-1.64 (m, 2H), 1.25 (t, *J* = 7.12 Hz, 3H), 1.18 (s, 6H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 177.7, 60.6, 45.5, 42.0, 38.0, 28.6, 25.3, 14.4.

HRMS (ESI^+) *m/z* calc'd for $C_9H_{17}ClO_2$ $[M+H]^+$ = 192.0917, found $[M+H]^+$ 193.0990

Ethyl 5-azido-2,2-dimethylpentanoate (13)

A round bottom flask was equipped with a magnetic stir bar and charged with NaN_3 (675 mg, 10.4 mmol), **12** (1.0 g, 5.2 mmol), and DMSO (6 mL). This was stirred overnight at 60 °C. The next day the reaction mixture allowed to warm to room temperature, diluted with 40 mL of water, and washed with diethyl ether (3 x 60 mL). The organic layers were washed with water (1 x 60 mL), brine (1 x 60 mL), then dried with $MgSO_4$, filtered, and solvent was removed by rotary evaporation to give the product as a clear liquid. (986 mg, 95%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 4.12-4.09 (q, *J* = 7.12 Hz, 2H), 3.24-3.22 (t, *J* = 6.48 Hz, 2H), 1.58-1.51 (m, 4H), 1.24-1.22 (t, *J* = 7.12 Hz, 3H), 1.16 (s, 6H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 177.7, 60.6, 51.9, 42.1, 37.8, 25.3, 24.8, 14.2.

HRMS (ESI^+) *m/z* calc'd for $C_9H_{17}N_3O_2$ $[M+Na]^+$ = 222.1213, found $[M+Na]^+$ 222.1221.

5-azido-2,2-dimethylpentanoic acid (14)

A round bottom flask was equipped with a magnetic stir bar and charged with **13** (1.0 g, 5.02 mmol), KOH (1.4 g, 25.1 mmol), and 2:1 EtOH:H_2O (9.8 mL). This was left stirring for 3 hours

at 80 °C. The reaction mixture was then warmed to room temperature, diluted with 20 mL of water, 30 mL of 1M H_3PO_4, and washed with diethyl ether (3 x 60 mL). The organic layers were washed with brine (1 x 60 mL), then dried with $MgSO_4$, filtered, and solvent was removed by rotary evaporation to give the product as an orange oil. (816 mg, 95%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 3.27-3.25 (t, J = 6.02 Hz, 2H), 1.60-1.58 (m, 4H), 1.21 (s, 6H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 183.5, 51.9, 42.0, 37.5, 25.2, 24.8.

HRMS (ESI^-) *m/z* calc'd for $C_7H_{13}N_3O_2$ $[M-H]^-$ = 170.0935, found $[M-H]^-$ 170.0938.

Cbz-G3-$(N_3)_8$ (15) - *Modified from literature procedures*[97]

An oven dried round bottom flask was equipped with a magnetic stir bar, purged under vacuum and backfilled with $Ar_{(g)}$ three times before addition of **14** (1.32 g, 7.72 mmol), CDI (1.47 g, 7.72 mmol) and dry EtOAc (3 mL). This was stirred at 50 °C for 15 minutes at which point a solution of Cbz-G3-$(OH)_8$ (0.25 g, 0.24 mmol) and CsF (0.31 g, 1.93 mmol) in 1 mL of dry EtOAc were added. The reaction mixture was stirred at 50 °C under $Ar_{(g)}$ for 3 days at which point MS showed full conversion to the product. The reaction was then quenched with 1 mL of water and stirred for 15 minutes, followed by dilution with 50 mL of EtOAc and then washed with 1M H_3PO_4 (3 x 50 mL), 10% Na_2CO_3 (3 x 50 mL), and brine (1 x 50 mL). The organic layers were dried with $MgSO_4$, filtered, and solvent was removed by rotary evaporation. The crude material was purified by flash chromatography using a 40 g silica Sorbtech EZ Flash column with 5-40% acetone in hexanes (loaded in DCM) and monitored at 205 nm. Fractions containing product were combined, concentrated by rotary evaporation and dried under vacuum to afford the product as a yellow oil. (503 mg, 92%)

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.36-7.29 (m, 5H), 6.40 (s, 1H), 5.20 (s, 1H), 5.02 (s, 2H), 4.28-4.12 (m, 28H), 3.28-3.20 (m, 20H), 1.58-1.48 (m, 36H), 1.29-1.26 (m, 21H), 1.16 (s, 48H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 176.9, 172.1, 171.7, 171.5, 136.9, 128.7, 128.4, 67.6, 66.7, 65.9, 65.8, 65.1, 51.8, 46.9, 46.8, 46.6, 42.3, 37.6, 25.2, 24.7, 17.9, 17.8, 17.7.

HRMS (ESI^+) *m/z* calc'd for $C_{103}H_{162}N_{26}O_{31}$ $[M+2Na]^{2+}$ = 1152.5842, found $[M+2Na]^{2+}$ 1152.5846.

Cbz-G4-$(N_3)_{16}$ (16)

An oven dried round bottom flask was equipped with a magnetic stir bar, purged under vacuum and backfilled with $Ar_{(g)}$ three times before addition of **14** (0.50 g, 2.93 mmol), NMI (820 µL, 10.3 mmol), dry MeCN (1 mL), and TCFH (0.82 g, 2.93 mmol). This was left to stir for 10 min at room temperature at which point a solution of Cbz-G4-$(OH)_{16}$ (0.18 g, 0.092 mmol) in 500 µL of dry MeCN was added. The mixture was left stirring at room temperature for 30 min at which point it was quenched with 500 µL of water and stirred for another 10 min. The reaction mixture was then diluted with 30 mL of EtOAc and washed with water (3 x 60 mL), 1M H_3PO_4 (3 x 60 mL), 10% Na_2CO_3 (3 x 60 mL), and brine (1 x 60 mL). The organic layers were then dried with $MgSO_4$, filtered, and concentrated under rotary evaporation. The crude material was then purified using a 12 g silica Sorbtech EZ Flash column with 5-60% acetone in hexanes (loaded in DCM), and monitored at 205 nm. Fractions containing product were combined, concentrated by rotary evaporation and dried under vacuum to afford the product as a yellow oil. (297 mg, 73%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.35-7.26 (m, 5H), 6.48 (s, 1H), 5.34 (s, 1H), 5.05 (s, 2H), 4.39-4.05 (m, 60H), 3.36-3.10 (m, 36H), 1.57-1.44 (m, 68H), 1.28-1.23 (m, 45H), 1.14 (s, 96H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 176.8, 172.0, 171.6, 171.4, 136.9, 128.7, 128.2, 68.0, 66.6, 66.0, 65.5, 65.0, 51.8, 46.9, 46.7, 46.6, 42.3, 37.6, 25.2, 24.8, 17.9, 17.7, 17.6.

HRMS (ESI^+) *m/z* calc'd for $C_{199}H_{314}N_{50}O_{63}$ $[M+2Na]^{2+}$ = 2229.1350, found $[M+2Na]^{2+}$ 2229.1336.

Cbz-G5-$(N_3)_{32}$ (17)

An oven dried round bottom flask was equipped with a magnetic stir bar, purged under vacuum and backfilled with $Ar_{(g)}$ three times before addition of **14** (0.72 g, 4.19 mmol), NMI (1.17 mL, 14.7 mmol), dry MeCN (2 mL), and TCFH (1.18 g, 4.19 mmol). This was left to stir for 15 min at room temperature at which point a solution of Cbz-G5-$(OH)_{32}$ (0.25 g, 0.065 mmol) in 500 µL of dry MeCN was added. The mixture was left stirring at room temperature for 1 hour at which point it was quenched with 500 µL of water and stirred for another 10 min. The reaction mixture was then diluted with 60 mL of EtOAc and washed with water (3 x 60 mL), 1M H_3PO_4 (3 x 60 mL), 10% Na_2CO_3 (3 x 60 mL), and brine (1 x 60 mL). The organic layers were then dried with $MgSO_4$, filtered, and concentrated under rotary evaporation. The crude material was then purified using a 40 g silica Sorbtech EZ Flash column with 5-60% acetone in hexanes (loaded in DCM), and monitored at 205 nm. Fractions containing product were combined, concentrated by rotary evaporation and dried under vacuum to afford the product as a yellow oil. (477 mg, 84%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.33-7.26 (m, 5H), 6.47 (s, 1H), 5.48 (s, 1H), 5.04 (s, 2H), 4.31-4.05 (m, 124H), 3.27-3.14 (m, 68H), 1.56-1.45 (m, 132H), 1.26-1.23 (m, 93H), 1.14 (s, 192H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 176.7, 172.1, 171.6, 171.4, 136.9, 128.7, 128.3, 66.6, 66.0, 65.4, 65.3, 65.0, 51.8, 46.9, 46.8, 46.7, 42.4, 37.6, 25.4, 24.8, 18.0, 17.7, 17.76.

MALDI-MS *m/z* calc'd for $C_{391}H_{618}N_{98}O_{127}$ $[M+H]^+$ = 8719.49, found $[M+H]^+$ 8691.98.

mPEG-350-OMs (18a)

Using general procedure 3, mPEG-350-OH (10.0 g, 28.6 mmol) was dissolved in 190 mL of DCM followed by addition of TEA (15.9 mL, 114 mmol), MsCl (11.1 mL, 143 mmol), and then stirred for 15 min. After work-up the product was dried under vacuum to afford a yellow liquid. (12.03 g, 91%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 4.37-4.35 (m, 2H), 3.76-3.74 (m, 2H), 3.66-3.62 (m, 24H), 3.55-3.52 (m, 2H), 3.36 (s, 3H), 3.07 (s, 3H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 72.1, 70.8, 70.7, 69.5, 69.2, 59.2, 37.9.

mPEG-1000-OMs (18b)

Using general procedure 3, mPEG-1000-OH (10.0 g, 10 mmol) was dissolved in 65 mL of DCM followed by addition of TEA (5.6 mL, 40 mmol), MsCl (3.9 mL, 50 mmol) and then stirred for 1 hour. After work-up the product was dried under vacuum to afford a yellow waxy solid. (10.13 g, 94%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 4.36-4.35 (m, 2H), 3.75-3.73 (m, 2H), 3.65-3.61 (m, 82H), 3.52-3.51 (m, 2H), 3.35 (s, 3H), 3.06 (s, 3H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 72.1, 70.8, 69.5, 69.2, 59.2, 37.9.

mPEG-2000-OMs (18c)

Using general procedure 3, mPEG-2000-OH (10.0 g, 5.0 mmol) was dissolved in 35 mL of DCM followed by addition of TEA (2.8 mL, 20.0 mmol), MsCl (1.9 mL, 25.0 mmol) and then stirred for 1 hour. After work-up the product precipitated into diethyl ether, filtered, then dried under vacuum to afford a white powder. (9.46 g, 90%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 4.38-4.37 (m, 2H), 3.77-3.75 (m, 2H), 3.67-3.61 (m, 178H), 3.55-3.53 (m, 2H), 3.37 (s, 3H), 3.08 (s, 3H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 72.1, 70.8, 69.5, 69.2, 59.2, 37.9.

mPEG-350-N_3 (19a) - *Modified from literature procedures*[105,108]

A round bottom flask was equipped with a magnetic stir bar and charged with mPEG-350-OMs (5.0 g, 10.8 mmol), NaN_3 (1.4 g, 21.6 mmol), and EtOH (110 mL). This was stirred overnight at reflux. The next day, the reaction was cooled to room temperature and the solvent was removed

through rotary evaporation. The residue was dissolved in THF and the precipitate was removed by filtration. THF was removed through rotary evaporation and residual BHT was removed by passing the product through a silica plug with ~4 column volumes of 100% DCM. The product was then collected by flushing the column with ~4 column volumes of 20% MeOH in DCM, then ~2 column volumes of 100% MeOH. The solvent was removed by rotary evaporation followed by drying under vacuum overnight to afford the product as a yellow liquid. (4.08 g, 92%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 3.68-3.63 (m, 26H), 3.55-3.54 (m, 2H), 3.39-3.37 (m, 5H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 72.1, 70.9, 70.8, 70.7, 70.2, 59.2, 50.9.

mPEG-1000-N_3 (19b) - *Modified from literature procedures*[105,108]

A round bottom flask was equipped with a magnetic stir bar and charged with mPEG-1000-OMs (5.0 g, 4.63 mmol), NaN_3 (602 mg, 9.26 mmol), and EtOH (50 mL). This was stirred overnight at reflux. The next day, the reaction was cooled to room temperature and the solvent was removed through rotary evaporation. The residue was dissolved in THF and the precipitate was removed by filtration. THF was removed by rotary evaporation and residual BHT was removed by passing the product through a silica plug with ~4 column volumes of 100% DCM. The product was then collected by flushing the column with ~4 column volumes of 20% MeOH in DCM, then ~2 column volumes of 100% MeOH. The solvent was removed by rotary evaporation followed by drying under vacuum overnight to afford the product as a pale yellow waxy solid. (4.01 g, 84%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 3.68-3.63 (m, 86H), 3.55-3.54 (m, 2H), 3.40-3.38 (m, 5H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 72.1, 70.8, 70.7, 70.2, 59.2, 50.9.

mPEG-2000-N_3 (19c) - *Modified from literature procedures*[105,108]

A round bottom flask was equipped with a magnetic stir bar and charged with mPEG-2000-OMs (2.6 g, 1.26 mmol), NaN_3 (164 mg, 2.53 mmol), and DMF (15 mL). This was stirred overnight at

80 °C. The reaction was then cooled to room temperature then filtered through neutral alumina and washed with EtOAc. Solvent was removed by rotary evaporation and the product was precipitated into diethyl ether, filtered and then dried under vacuum to afford a white powder. (2.09 g, 81%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 3.68-3.62 (m, 181H), 3.55-3.54 (m, 2H), 3.39-3.37 (m, 5H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 72.1, 70.8, 70.7, 70.6, 59.2, 50.8.

mPEG-350-NH_2 (20a)

Using general procedure 4, PEG-350-N_3 (1.219 g, 2.98 mmol) was dissolved in 60 mL of 1:1 DCM:MeOH followed by addition of $Pd(OH)_2/C$ (122 mg, 10 wt%). After filtration with a syringe filter the solvent was removed by rotary evaporation and the product was dried under vacuum to afford a yellow liquid. (1.12 g, 98%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 3.66-3.56 (m, 26H), 3.54-3.48 (m, 2H), 3.34 (s, 3H), 2.87-2.83 (m, 2H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 72.1, 72.0, 71.9, 71.4, 71.1, 70.7, 70.6, 70.5, 70.4, 70.3, 59.2, 41.4, 31.1.

mPEG-1000-NH_2 (20b)

Using general procedure 4, PEG-1000-N_3 (1.255 g, 1.22 mmol) was dissolved in 25 mL of 1:1 DCM: MeOH followed by addition of $Pd(OH)_2/C$ (126 mg, 10 wt%). After filtration with a syringe filter the solvent was removed by rotary evaporation and the product was dried under vacuum to afford a yellow wax. (1.19 g, 97%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 3.75-3.60 (m, 86H), 3.54-3.52 (m, 2H), 3.36 (s, 3H), 3.02-2.99 (m, 2H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 72.0, 70.7, 70.6, 70.5, 70.4, 70.3, 70.2, 59.1, 41.1, 31.0.

mPEG-2000-NH_2 (20c)

Using general procedure 4, PEG-2000-N_3 (1.18 g, 0.58 mmol) was dissolved in 12 mL of 1:1 DCM: MeOH followed by addition of $Pd(OH)_2/C$ (118 mg, 10 wt%). After filtration with a syringe filter the product was precipitated into diethyl ether, filtered, and dried under vacuum to afford a white powder. (976 mg, 84%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 3.94-3.92 (m, 2H), 3.77-3.75 (m, 2H), 3.72-3.60 (m, 178H), 3.55-3.51 (m, 2H), 3.38 (s, 3H), 3.19-3.17 (m, 2H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 72.1, 70.7, 70.6, 70.5, 70.4, 70.3, 70.2, 59.2, 40.7, 31.1.

DBCO-NHS (21) - *Modified from literature procedures*[88]

A round bottom flask was equipped with a magnetic stir bar and charged with DBCO-COOH (600 mg, 1.97 mmol), DCM (30 mL), NHS (339 mg, 2.95 mmol), and EDC (565 mg, 2.95 mmol). This was stirred at room temperature for 2 hours. The crude mixture was then purified by flash chromatography using a 12 g silica Sorbtech EZ Flash column with 40-100% EtOAc in hexanes monitored at 254 nm. Fractions containing product were collected, and solvent was removed by rotary evaporation. The resulting oil was dissolved in 2 mL of DCM and precipitated into 50 mL of stirring hexanes. The product was then filtered and dried under vacuum to afford a white powder. (586 mg, 74%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.70-7.69 (m, 1H), 7.43-7.36 (m, 5H), 7.33-7.30 (m 1H), 7.27-7.25 (m, 1H), 5.18 (d, J = 13.90 Hz, 1H), 3.69 (d, J = 13.90 Hz, 1H), 3.00-2.95 (m, 1H), 2.84-2.79 (m, 5H), 2.70-2.62 (m, 1H), 2.11-2.07 (m, 1H).

HRMS (ESI^+) *m/z* calc'd for $C_{23}H_{18}N_2O_5$ $[M+H]^+$ = 403.1294, found $[M+H]^+$ 403.1296.

mPEG-350-DBCO (22a)

Using general procedure 5, mPEG-NH_2 (1.0 g, 2.61 mmol) was dissolved in 10 mL of DCM, followed by addition of DBCO-NHS (1.26 g, 3.13 mmol) and TEA (0.73 mL, 5.22 mmol).

The next day the reaction was directly purified by flash chromatography first using a 40 g neutral alumina column with 20% MeOH in DCM. Fractions containing product were collected, concentrated by rotary evaporation, and then purified again using a 120 g silica Sorbtech EZ Flash column with 0-5% MeOH in DCM. Fractions containing product were collected, solvent was removed by rotary evaporation, and then dried under vacuum to afford the product as a yellow oil. (1.489 g, 85%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.68-7.67 (m, 1H), 7.53-7.51 (m, 1H), 7.41-7.34 (m, 4H), 7.31-7.28 (m, 1H), 7.25-7.24 (m, 1H), 6.23 (s, 1H), 5.15 (d, *J* = 13.89 Hz, 1H), 3.67-3.42 (m, 29H), 3.37 (s, 3H), 3.34-3.32 (m, 2H), 2.84-2.78 (m, 1H), 2.49-2.44 (m, 1H), 2.19-2.15 (m, 1H), 1.97-1.92 (m, 1H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 172.4, 172.2, 151.6, 148.3, 132.4, 129.5, 128.8, 128.3, 128.2, 127.8, 127.2, 125.6, 123.4, 122.6, 114.8, 108.1, 72.1, 70.8, 70.7, 70.6, 70.4, 69.9, 59.2, 55.6, 39.4, 31.3, 30.3.

mPEG-1000-DBCO (22b)

Using general procedure 5, mPEG-NH_2 (1.187 g, 1.19 mmol) was dissolved in 5 mL of DCM, followed by addition of DBCO-NHS (573 mg, 1.42 mmol) and TEA (0.33 mL, 2.37 mmol). The next day the reaction was directly purified by flash chromatography first using a 40 g neutral alumina column with 20% MeOH in DCM. Fractions containing product were collected, concentrated by rotary evaporation, and then purified again using a 120 g silica Sorbtech EZ Flash column with 0-5% MeOH in DCM. Fractions containing product were collected, solvent was

removed by rotary evaporation, and then dried under vacuum to afford the product as a yellow wax. (1.287 g, 84%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.68-7.67 (m, 1H), 7.53-7.51 (m, 1H), 7.41-7.34 (m, 4H), 7.31-7.28 (m, 1H), 7.26-7.24 (m, 1H), 6.27 (s, 1H), 5.15 (d, *J* = 13.89 Hz, 1H), 3.77-3.42 (m, 87H), 3.38 (s, 3H), 3.35-3.32 (m, 2H), 2.84-2.78 (m, 1H), 2.49-2.44 (m, 1H), 2.20-2.15 (m, 1H), 1.97-1.92 (m, 1H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 172.2, 172.1, 151.5, 148.1, 132.3, 129.4, 128.7, 128.2, 128.1, 127.7, 127.0, 125.4, 123.2, 122.5, 114.7, 108.0, 72.0, 70.6, 70.5, 70.2, 69.8, 59.0, 55.5, 39.2, 31.2, 30.2.

mPEG-2000-DBCO (22c)

Using general procedure 5, mPEG-NH_2 (600 mg, 0.30 mmol) was dissolved in 1.2 mL of DCM, followed by addition of DBCO-NHS (144 mg, 0.36 mmol) and TEA (83 µL, 0.60 mmol). The next day the reaction was concentrated by rotary evaporation, precipitated into diethyl ether, and then dried under vacuum to afford the product as a white powder. (528 mg, 79%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.68-7.66 (m, 1H), 7.53-7.51 (m, 1H), 7.40-7.33 (m, 4H), 7.31-7.28 (m, 1H), 7.26-7.24 (m, 1H), 6.36 (s, 1H), 5.15 (d, *J* = 13.89 Hz, 1H), 3.76-3.42 (m, 185H), 3.38 (s, 3H), 3.35-3.32 (m, 2H), 2.83-2.78 (m, 1H), 2.49-2.44 (m, 1H), 2.19-2.15 (m, 1H), 1.97-1.92 (m, 1H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 172.3, 172.2, 151.6, 148.2, 132.4, 129.5, 128.7, 128.3, 128.2, 127.8, 127.1, 125.6, 123.3, 122.6, 114.8, 108.0, 72.1, 70.6, 70.3, 69.9, 59.1, 55.6, 39.4, 31.3, 30.3.

Cbz-G3-(mPEG-350)$_8$ (23a)

Using general procedure 6, Cbz-G3-(N_3)$_8$ (100 mg, 0.04 mmol) was dissolved in 2 mL of DCM, followed by addition of mPEG-350-DBCO (356 mg, 0.53 mmol). The mixture was purified using

3.5 kDa MWCO dialysis tubing and the product was afforded as a viscous orange oil. (234 mg, 69%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.68-7.00 (m, 69H), 6.36 (d, *J* = 76.8 Hz, 8H), 6.02 (dd, *J* = 43.1, 16.7 Hz, 8H), 5.02 (s, 2H), 4.42-4.08 (m, 44H), 3.74-3.46 (m, 240H), 3.36-3.28 (m, 40H), 2.39-1.09 (m, 137H).

Cbz-G3-(mPEG-1000)$_8$ (23b)

Using general procedure 6, Cbz-G3-(N_3)$_8$ (70 mg, 0.03 mmol) was dissolved in 2 mL of DCM, followed by addition of mPEG-1000-DBCO (478 mg, 0.37 mmol). The mixture was purified using 50 kDa MWCO dialysis tubing and the product was afforded as a viscous orange oil. (198 mg, 51%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.61-7.01 (m, 69H), 6.33 (d, *J* = 74.5 Hz, 8H), 6.02 (dd, *J* = 40.6, 16.2 Hz, 8H), 5.01 (s, 2H), 4.41-4.14 (m, 44H), 3.76-3.46 (m, 720H), 3.37-3.29 (m, 40H), 2.39-1.08 (m, 137H).

Cbz-G3-(mPEG-2000)$_8$ (23c)

Using general procedure 6, Cbz-G3-(N_3)$_8$ (40 mg, 0.018 mmol) was dissolved in 2 mL of DCM, followed by addition of mPEG-2000-DBCO (479 mg, 0.21 mmol). The mixture was purified using 100 kDa MWCO dialysis tubing and the product was afforded as a fluffy white powder. (148 mg, 40%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.60-7.00 (m, 69H), 6.31 (d, *J* = 69.5 Hz, 8H), 6.02 (dd, *J* = 41.6, 15.9 Hz, 8H), 5.01 (s, 2H), 4.41-4.12 (m, 44H), 3.76-3.46 (m, 1520H), 3.37-3.28 (m, 40H), 2.38-1.08 (m, 137H).

Cbz-G4-(mPEG-350)$_{16}$ (24a)

Using general procedure 6, Cbz-G4-(N_3)$_{16}$ (10 mg, 0.003 mmol) was dissolved in 1 mL of DCM, followed by addition of mPEG-350-DBCO (36 mg, 0.054 mmol). Upon completion, the solvent was removed to afford the crude material as a viscous yellow oil, and was analyzed without purification.

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.68-7.00 (m, 133H), 6.40 (d, *J* = 81.4 Hz, 16H), 6.01 (dd, *J* = 33.8, 16.7 Hz, 16H), 5.00 (s, 2H), 4.41-4.13 (m, 92H), 3.73-3.42 (m, 480H), 3.38-3.32 (m, 80H), 1.85-1.08 (m, 273H).

Cbz-G4-(mPEG-1000)$_{16}$ (24b)

Using general procedure 6, Cbz-G4-(N_3)$_{16}$ (10 mg, 0.003 mmol) was dissolved in 1 mL of DCM, followed by addition of mPEG-1000-DBCO (70 mg, 0.054 mmol). Upon completion, the solvent was removed to afford the crude material as a viscous yellow oil, and was analyzed without purification.

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.68-7.00 (m, 133H), 6.37 (d, *J* = 86.4 Hz, 16H), 6.01 (dd, *J* = 30.9, 16.4 Hz, 16H), 4.99 (s, 2H), 4.39-4.13 (m, 92H), 3.76-3.41 (m, 1440H), 3.37-3.28 (m, 80H), 1.86-1.08 (m, 273H).

Cbz-G4-(mPEG-2000)$_{16}$ (24c)

Using general procedure 6, Cbz-G4-(N_3)$_{16}$ (8 mg, 0.002 mmol) was dissolved in 1 mL of DCM, followed by addition of mPEG-2000-DBCO (98 mg, 0.044 mmol). Upon completion, the solvent was removed to afford the crude material as a viscous yellow oil, and was analyzed without purification.

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.67-6.99 (m, 133H), 6.35 (d, *J* = 83.4 Hz, 16H), 6.00 (dd, *J* = 29.0, 16.4 Hz, 16H), 4.99 (s, 2H), 4.39-4.12 (m, 92H), 3.76-3.41 (m, 3040H), 3.37-3.27 (m, 80H), 1.91-1.07 (m, 273H).

Cbz-G5-(mPEG-350)$_{32}$ (25a)

Using general procedure 6, Cbz-G5-$(N_3)_{32}$ (10 mg, 0.0014 mmol) was dissolved in 1 mL of DCM, followed by addition of mPEG-350-DBCO (37 mg, 0.055 mmol). Upon completion, the solvent was removed to afford the crude material as a viscous yellow oil, and was analyzed without purification.

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.68-6.99 (m, 261H), 6.53 (d, *J* = 101.7 Hz, 32H), 6.03-5.96 (m, 32H), 5.00 (s, 2H), 4.39-4.12 (m, 188H), 3.73-3.42 (m, 960H), 3.38-3.24 (m, 160H), 1.85-1.06 (m, 545H).

Cbz-G5-(mPEG-1000)$_{32}$ (25b)

Using general procedure 6, Cbz-G5-$(N_3)_{32}$ (8 mg, 0.001 mmol) was dissolved in 1 mL of DCM, followed by addition of mPEG-1000-DBCO (57 mg, 0.044 mmol). Upon completion, the solvent was removed to afford the crude material as a viscous yellow oil, and was analyzed without purification.

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.68-6.98 (m, 261H), 6.51 (d, *J* = 101.2 Hz, 32H), 6.02-5.95 (m, 32H), 5.00 (s, 2H), 4.38-4.10 (m, 188H), 3.76-3.42 (m, 2280H), 3.37-3.23 (m, 160H), 1.86-1.06 (m, 545H).

Cbz-G5-(mPEG-2000)$_{32}$ (25c)

Using general procedure 6, Cbz-G5-$(N_3)_{32}$ (5 mg, 0.0006 mmol) was dissolved in 1 mL of DCM, followed by addition of mPEG-2000-DBCO (62 mg, 0.028 mmol). Upon completion, the solvent

was removed to afford the crude material as a viscous yellow oil, and was analyzed without purification.

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.68-6.98 (m, 261H), 6.49 (d, *J* = 97.0 Hz, 32H), 6.01-5.94 (m, 32H), 4.99 (s, 2H), 4.38-4.10 (m, 188H), 3.76-3.42 (m, 6080H), 3.37-3.23 (m, 160H), 1.79-1.05 (m, 545H).

NH_2-G3-(mPEG-350)$_8$ (26a) – *Prepared according to literature procedures*[93]

A round bottom flask was equipped with a magnetic stir bar, and charged with Cbz-G3-(mPEG-350)$_8$ (23 mg, 0.003 mmol), $Pd(OH)_2/C$ (2 mg, 10 wt%), and 1:1 MeOH:DCM (1 mL). The reaction vessel was purged under vacuum and backfilled with hydrogen gas three times while stirring, and was then left stirring at room temperature overnight under hydrogen gas. The next day the reaction mixture was filtered using a 0.2 μm syringe filter, concentrated by rotary evaporation and then dried under vacuum to afford the product as a pale yellow oil. (20 mg, 87%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.68-7.00 (m, 69H), 6.45-6.28 (m, 8H), 6.02 (dd, *J* = 43.1, 16.7 Hz, 8H), 4.42-4.08 (m, 44H), 3.74-3.46 (m, 240H), 3.36-3.28 (m, 40H), 2.39-1.09 (m, 137H).

Chapter 3 - Synthesis and Characterization of DBCO Core, PEGylated Dendrimers Using A Convergent Approach

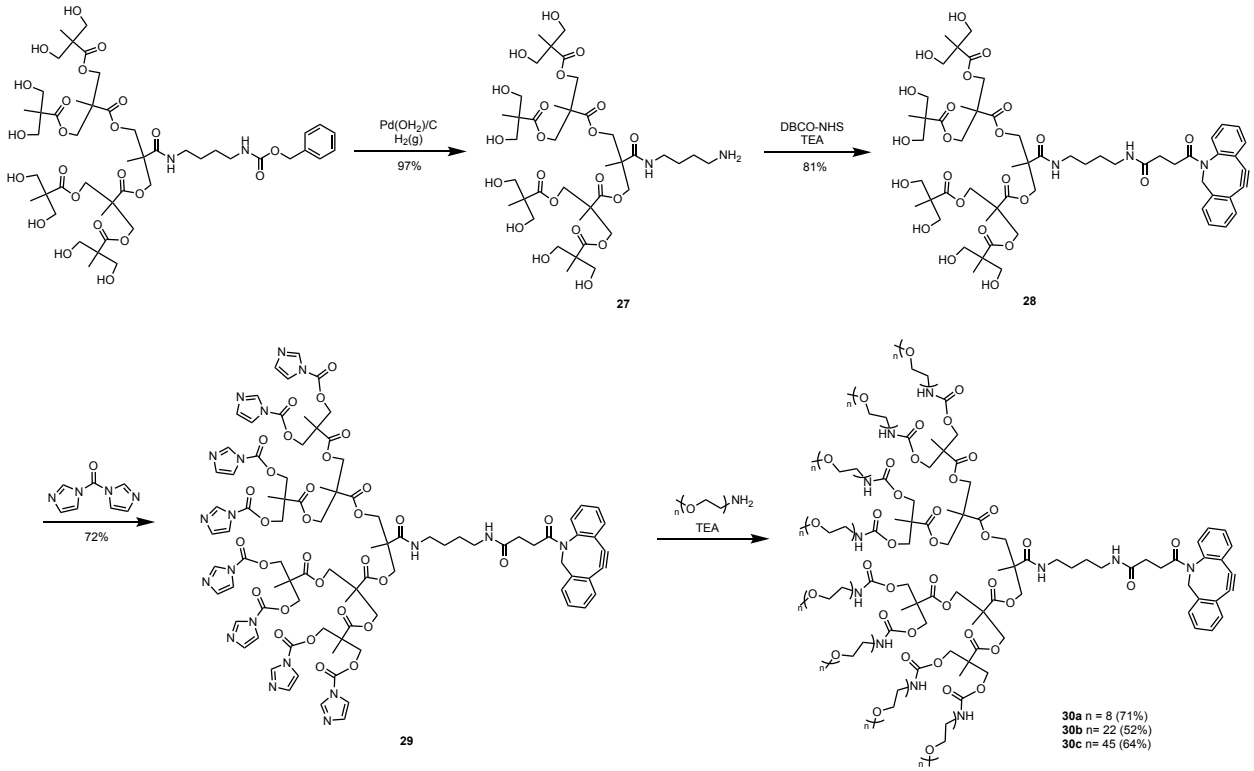
3.1 Overview

In this chapter, a convergent synthesis strategy was developed to prepare the dendrimers that were set out to be prepared in Chapter 2. This synthetic approach was developed to circumvent the previous issue of complete core isolation as a result of PEG chain entanglement at the dendrimer periphery. In this approach, a series of DBCO core, G3 PEGylated Bis-MPA dendrimers, or, "outer dendrons" were first prepared following literature procedures. Next, a series of "inner dendrons" consisting of an amine core and an azide periphery were synthesized using similar chemistry outlined in Chapter 2. A key difference in this synthetic approach is the protecting group used at the core, and its removal prior to PEGylation. Upon preparation of the inner and outer dendrons, the G4 and G5 dendrimers were prepared convergently using SPAAC chemistry. Coupling of the free amine at the core of these dendrimers with an activated DBCO-NHS ester was the final synthetic step to obtain the DBCO core, PEGylated dendrimers. All dendrimers were further characterized using MALDI-TOF MS to better approximate their molecular weight. DLS measurements were taken to further understand the relative size of each dendrimer, however, the results were inconclusive.

3.2 Synthesis of Inner and Outer Dendrons

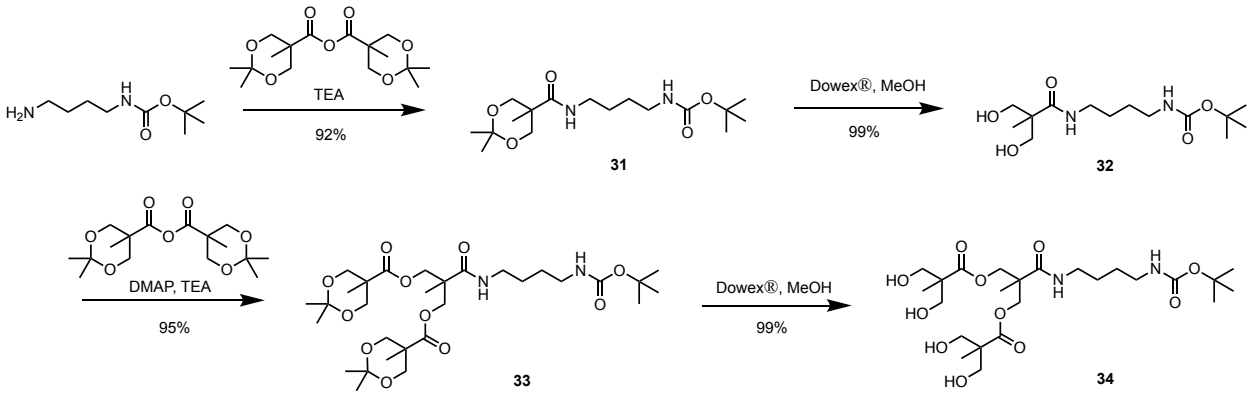
The outer dendrons were first prepared as outlined in Scheme 3.1 following literature procedures.[88] This first involved deprotecting the core of Cbz-G3-$(OH)_8$, previously prepared in Chapter 2, through a hydrogenolysis reaction with a $Pd(OH)_2/C$ catalyst. This afforded a free amine at the core which then coupled with DBCO-NHS to yield a DBCO core dendrimer. The periphery

was then activated using CDI which allowed for easy PEGylation of the dendrimers using the amine-terminated PEG chains (PEG 350, 1000, and 2000) which were prepared in chapter 2. This yielded three third-generation dendrimers each with a reactive DBCO core, and a different molecular weight PEG chain at the periphery.



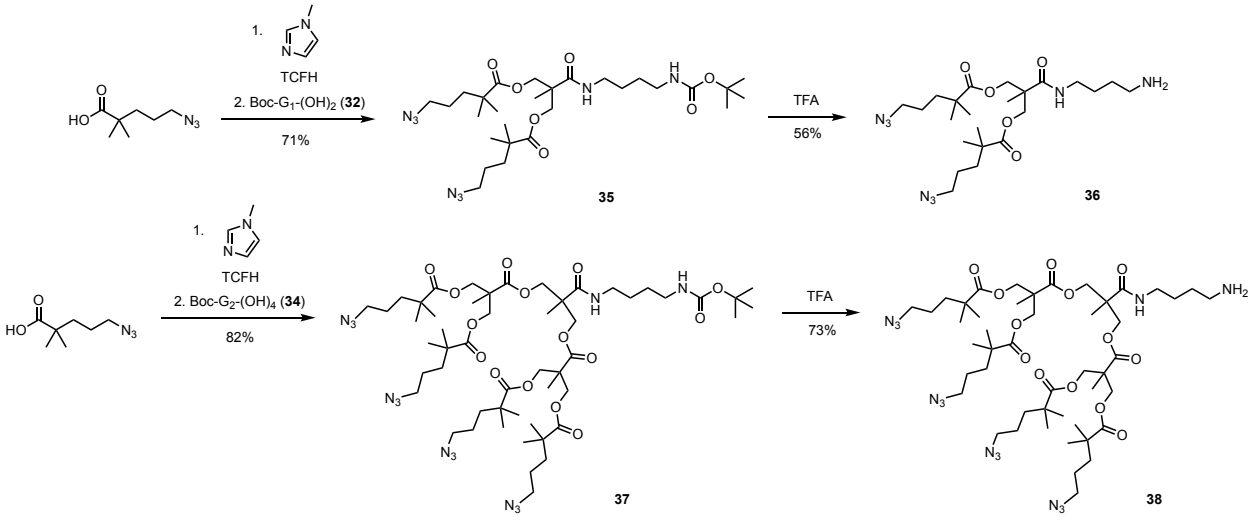
Scheme 3.1: Synthesis of DBCO-G3-$(PEG)_8$ derivatives.

The inner dendrons were then prepared as shown in Scheme 3.2. This was completed in an exact manner as described in Chapter 2, however, a Boc protected core was used instead of a Cbz protected core. This was chosen as the protecting group because Boc removal with TFA will not disrupt the azide functionalities that will be installed later. Had we opted for another Cbz protected core, the peripheral azide groups would reduce to amines upon hydrogenolysis when deprotecting the core.



Scheme 3.2: Synthesis of G1 and G2 Boc core, Bis-MPA dendrons.

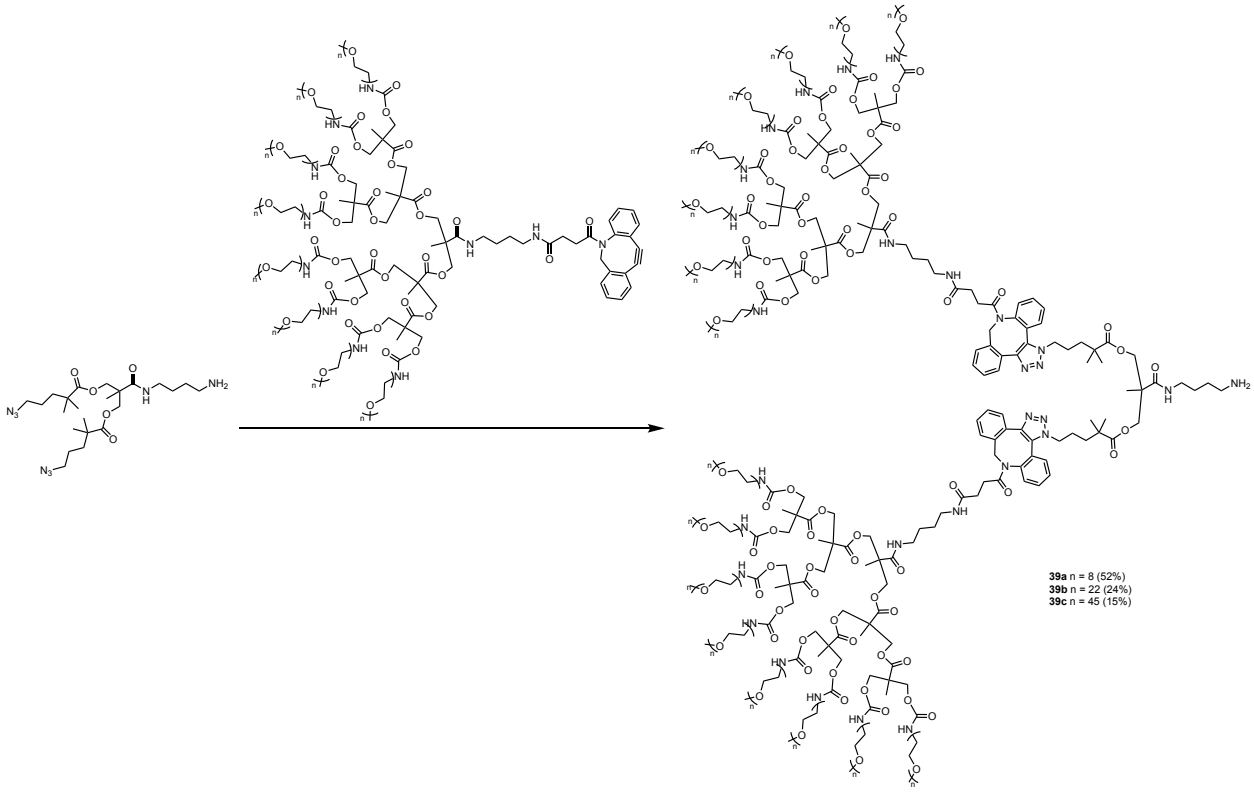
The alcohol periphery G1 and G2 inner dendrons were then functionalized with azide groups as illustrated in Scheme 3.3. This was completed using identical chemistry as described in Chapter 2 in which the neopentyl azide linker prepared was first activated with TCFH and NMI, and then appended to the periphery of the dendrimers in a one-pot synthesis. The Boc protected cores were then removed using TFA to yield amine core, azide periphery dendrons. We chose to remove the core prior to PEGylation so that only one final synthetic step would be needed to prepare the DBCO-core dendrimers, post-PEGylation. These inner dendrons can click with the outer dendrons synthesized to construct the G4 and G5 PEGylated dendrimers.



Scheme 3.3: Synthesis of NH_2-G1-$(N_3)_2$ and NH_2-G2-$(N_3)_4$.

3.3 Dendrimer Assembly Using SPAAC Chemistry

With the inner (azide periphery) and outer (DBCO-core) dendrons in hand, G4 PEGylated dendrimers were easily prepared as shown in Scheme 3.4. In an attempt to minimize the purification, an exact 1:2 ratio of NH_2-G1-$(N_3)_2$: DBCO-G3-$(PEG)_8$ was used as there were two peripheral azide groups for every DBCO core dendron. This also reduced the amount of outer dendron that would be wasted to the purification process.



Scheme 3.4: Synthesis of NH_2-G4-$(mPEG)_{16}$ derivatives.

To prepare these, a precise solution of the outer dendron was prepared and then added in small aliquots to a solution of the inner dendron. Since a precise concentration of outer dendron was prepared we could approximate how much of it would be needed to obtain an exact 1:2 ratio of inner dendron : outer dendron. Furthermore, adding the outer dendron in small aliquots helped to prevent exceeding this exact ratio. After each aliquot was added, the reaction was left to stir at

room temperature for 20 minutes, and an FTIR measurement was made to observe for disappearance of the azide peak at ~ 2100 cm^{-1}. If residual azide was still present, another aliquot of outer dendron was added, left to react, and then monitored again by FTIR. This process was repeated until complete disappearance of the azide peak was observed at which point the reaction was deemed finished.

^{1}H NMR was used to confirm that the inner azide periphery dendron was fully functionalized with the outer DBCO core dendron. Similar to what was reported in Chapter 2, we observe a change in chemical shift in one of the methylene protons in the cyclooctyne from 5.17 ppm to ~6 ppm, as seen in Figure 3.1 below. As expected, this new peak roughly appears as a doublet of doublets and integrates to 2H as two DBCO moieties were clicked onto the periphery. An exact doublet of doublets splitting pattern is not observed since these protons constitute <1% to the total polymer, making their corresponding signal very weak. We also observe the expected integrations for amide protons that link the PEG chain to the outer dendron. Pre-click (Figure 3.1a), these protons are observed at ~5.5 ppm with an integration of 8H as 8 PEG chains are on the periphery of the G3 dendrimer. Post-click (Figure 3.1b), this integral value doubles to 16H as we now have 16 PEG chains on the periphery of the dendrimer. These critical NMR integral values corroborate the earlier FTIR data in that full PEGylation of the dendrimer was achieved.

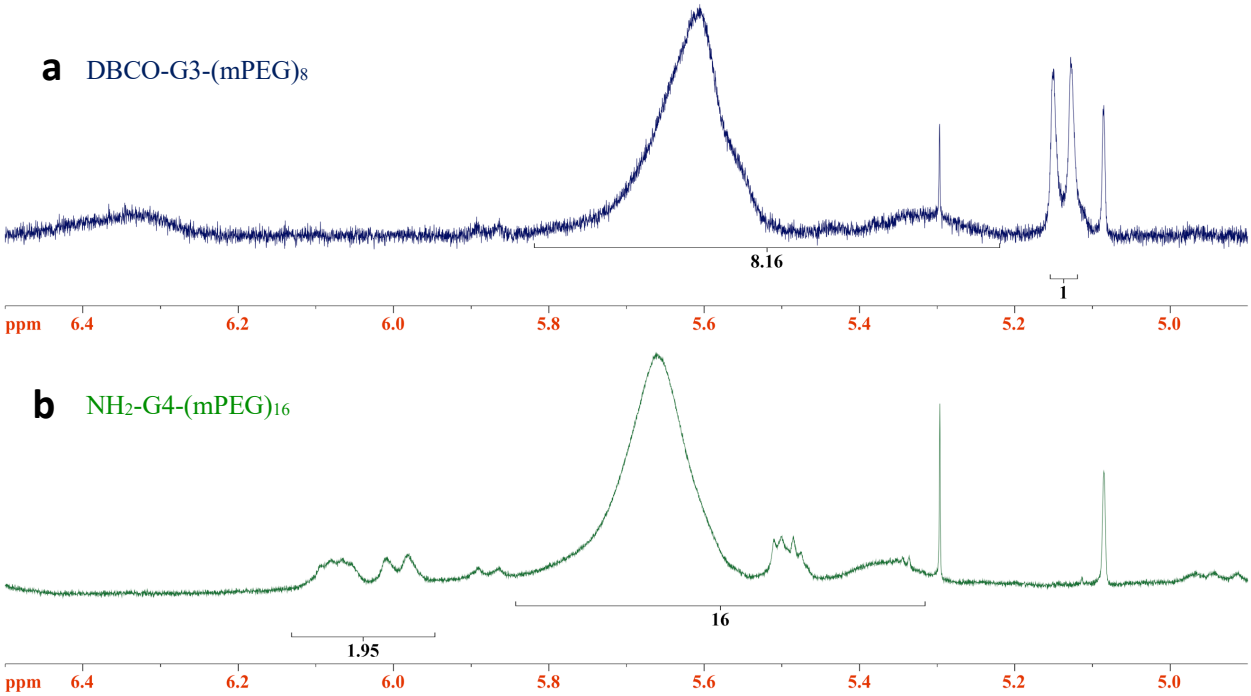
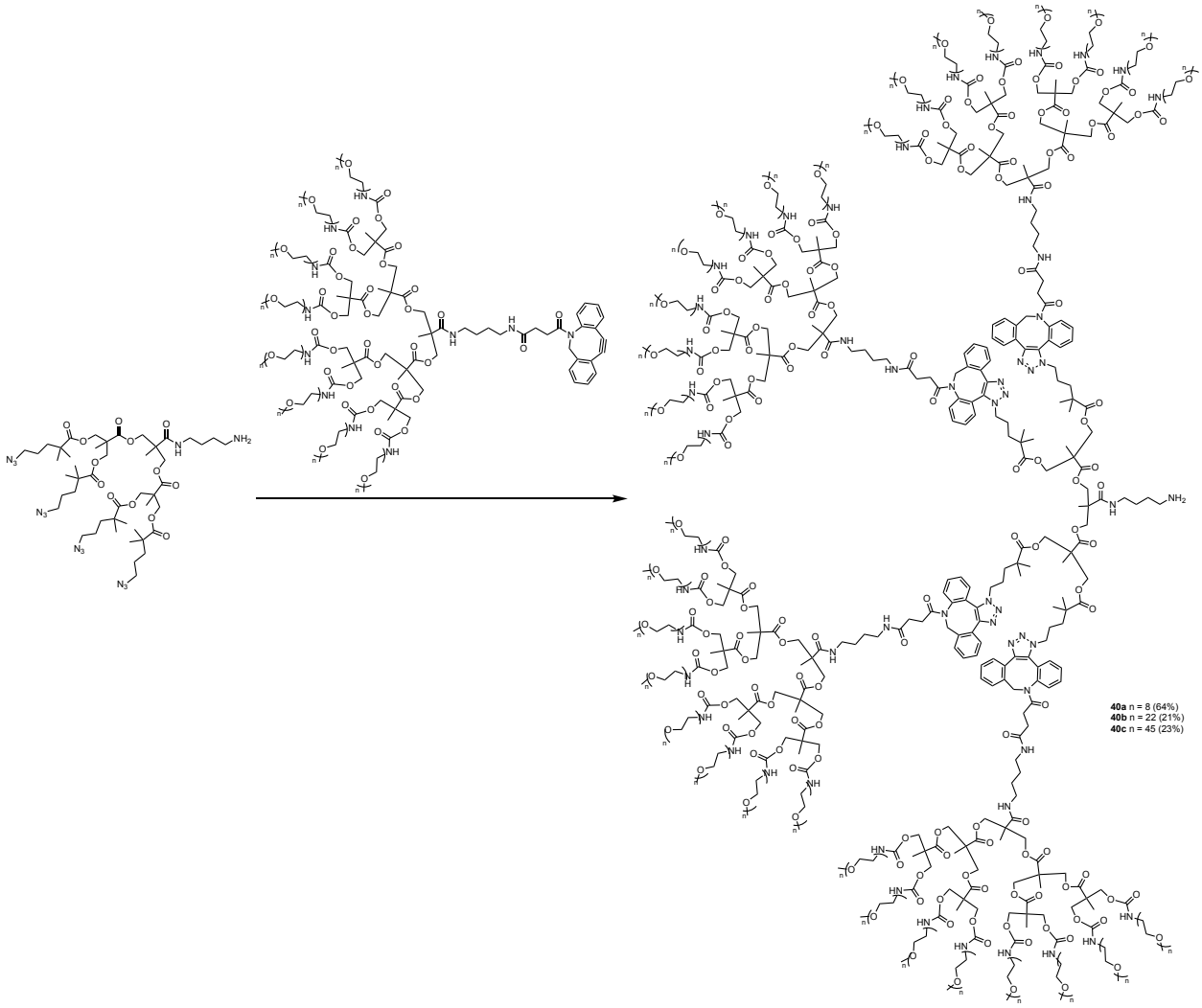


Figure 3.1: ^{1}HNMR spectra of: (a) a DBCO-G3-$(PEG)_8$ derivative (pre-click), (b) a NH_2-G4-$(PEG)_{16}$ dendrimer (post-click), with critical integral values illustrating complete PEGylation.

This entire process was also implemented to prepare the G5 PEGylated dendrimers as shown in Scheme 3.5. However, this time the second generation azide periphery dendrimer was used as the inner dendron, and a 1:4 ratio of NH_2-G2-$(N_3)_4$: DBCO-G3-(PEG) was used as there are four peripheral azides for every DBCO core dendron.



Scheme 3.5: Synthesis of NH_2-G5-$(mPEG)_{32}$ derivatives.

The reaction was again monitored by FTIR, and ^1H NMR was used to confirm that all four peripheral azides were functionalized with DBCO moieties. As seen in Figure 3.2b, we can vaguely observe the expected doublet of doublets splitting pattern at ~6 ppm with an integration of 4H as 4 DBCO units were clicked on to the periphery. Again, a precise splitting pattern is not observed due to the weak signal intensity of these protons as they make up a negligible amount of the overall polymer. We also observe the expected integrations for amide protons that link the PEG chains to the outer dendron. Pre-click (Figure 3.2a), these protons are observed at ~5.5 ppm with

an integration of 8H and post-click (Figure 3.2b), this integral value quadruples to 32H as 32 PEG chains are on the periphery of the dendrimer.

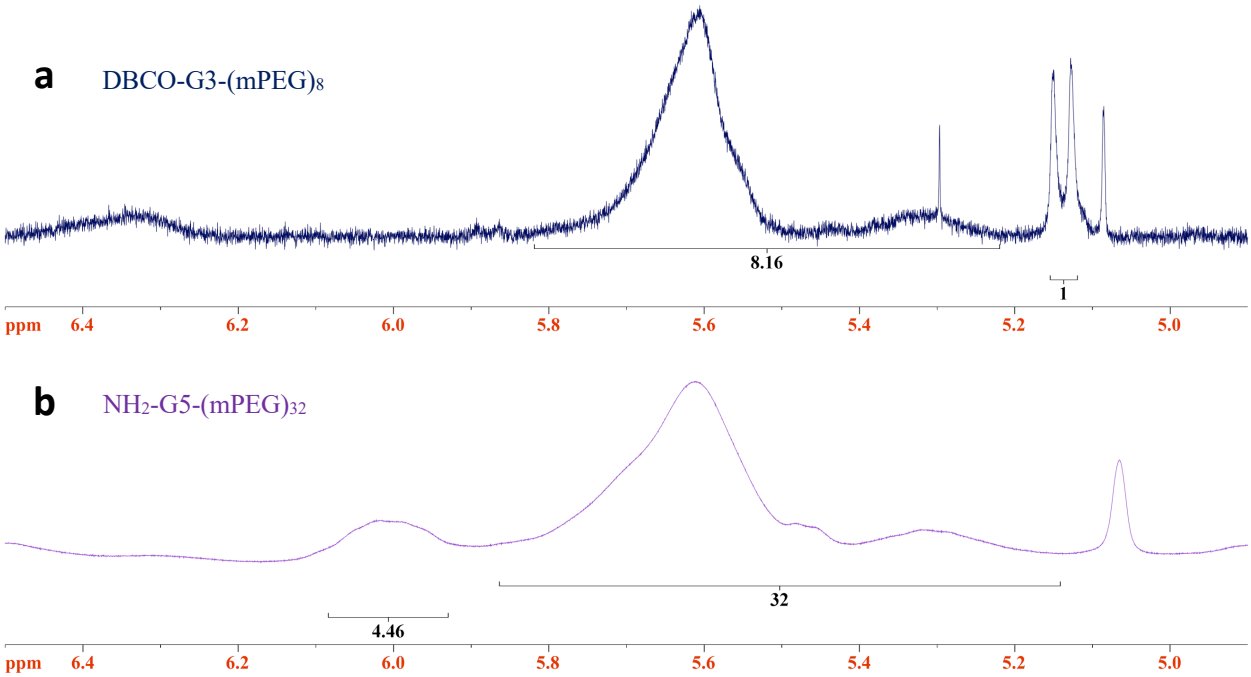
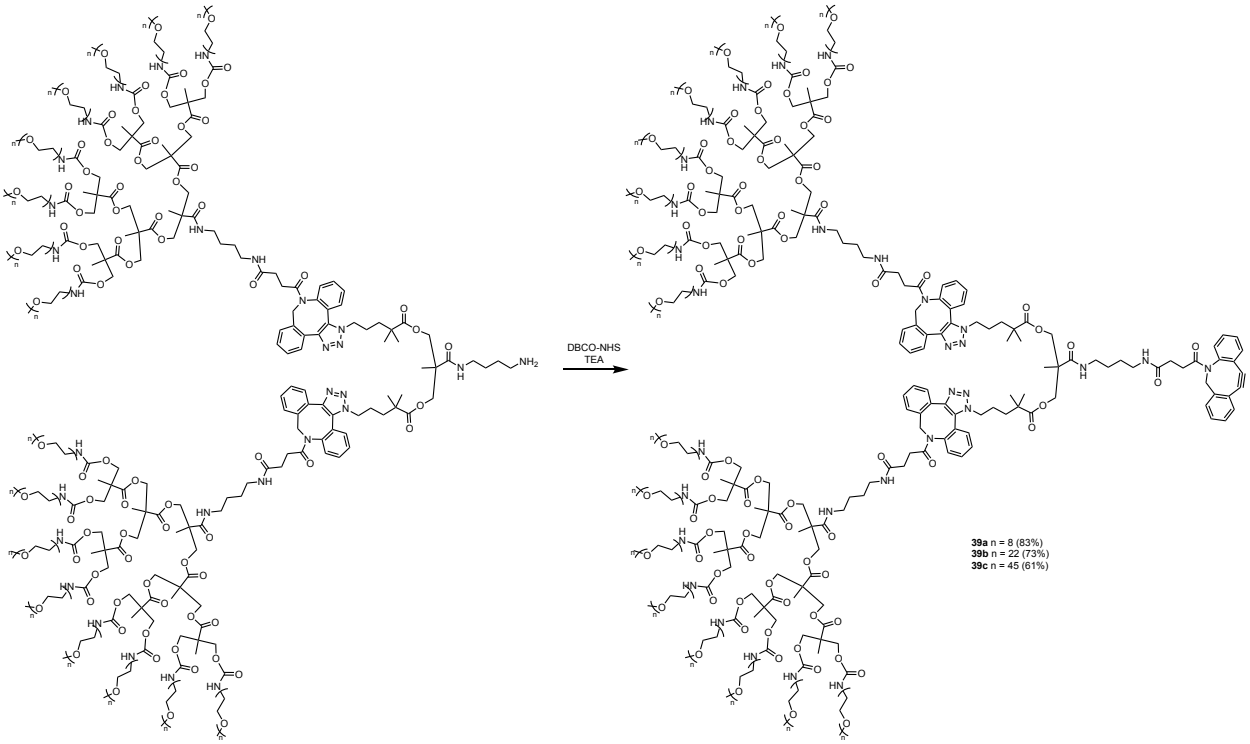


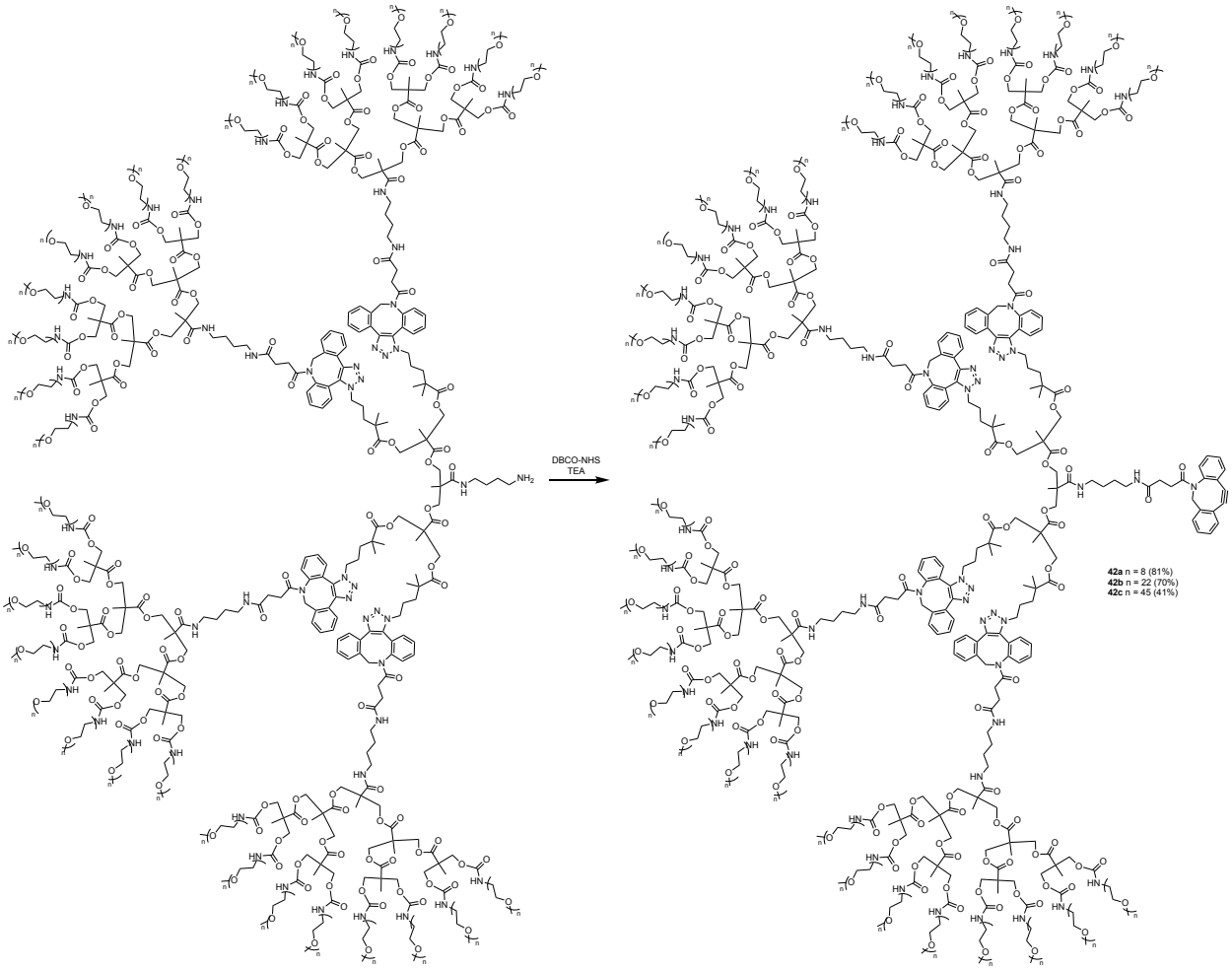
Figure 3.2: ^{1}HNMR spectra of: (a) a DBCO-G3-$(PEG)_8$ derivative (pre-click), (b) a NH_2-G5-$(PEG)_{32}$ dendrimer (post-click), with critical integral values illustrating complete PEGylation.

3.4 Core Functionalization

The final step in preparing the DBCO-core, PEGylated dendrimer library was to functionalize the core of the G4 and G5 PEGylated dendrimers. This was achieved through a DBCO-NHS coupling to the free amine at the core of each dendrimer as illustrated in Schemes 3.6 and 3.7.



Scheme 3.6: Synthesis of DBCO-G4-$(mPEG)_{16}$ derivatives.



Scheme 3.7: Synthesis of DBCO-G5-$(mPEG)_{32}$ derivatives.

Characterization of this synthetic step was rather challenging because addition of a small molecule onto a relatively large polymer makes subtle differences in both MALDI-TOF MS and ^{1}H NMR. A mass difference of ~ 300 Da (addition of a DBCO moiety) cannot be detected accurately using MALDI-TOF MS, especially for polydisperse polymers of higher molecular weight. Moreover, the ^{1}H NMR spectrum of the starting material is completely saturated with peaks at the chemical shifts in which the DBCO moiety would appear in the product making it near impossible to tell if the reaction worked. We would also be looking for just a few protons in a sample that already contains thousands of them, and these protons would likely be hidden in the baseline. Therefore, we opted to run test click reactions on a small molecule azide and used FTIR

to observe whether or not the azide peak was consumed. We hypothesized that if the azide peak was consumed upon reaction with our dendrimers, then it could be concluded that the DBCO-NHS coupling worked and the dendrimer did in fact have a DBCO unit at its core. Upon addition of ~1 eq. of dendrimer to 0.5 eq. of benzyl azide, complete consumption of the azide peak occurred in just 20 minutes of mixing for all G4 and G5 derivatives. This confirmed that all of the DBCO couplings to the core of the dendrimer were successful.

3.5 Dendrimer Characterization

DLS measurements were taken to obtain a better understanding of the overall size of each dendrimer, especially relative to one another. These results provided the volume average hydrodynamic diameter of each dendrimer prepared and are listed below in Table 3.1.

Table 3.1: Volume average hydrodynamic diameters of DBCO-core, PEGylated dendrimers as determined by DLS.

Dendrimer	Diameter (nm)
DBCO-G3-(PEG-350)$_8$	208 ± 5
DBCO-G3-(PEG-1000)$_8$	190 ± 17
DBCO-G3-(PEG-2000)$_8$	180 ± 1
DBCO-G4-(PEG-350)$_{16}$	198 ± 1
DBCO-G4-(PEG-1000)$_{16}$	117 ± 2
DBCO-G4-(PEG-2000)$_{16}$	160 ± 2
DBCO-G5-(PEG-350)$_{32}$	93 ± 1
DBCO-G5-(PEG-1000)$_{32}$	131 ± 1
DBCO-G5-(PEG-2000)$_{32}$	179 ± 2

This data obtained from DLS does not show any trend that is consistent with the perceived, or expected size of our dendrimers. When first analyzing the data it was confusing as to why the size of the smallest analog, DBCO-G3-(PEG-350)$_8$, was being determined as the largest of the

bunch. Furthermore, when comparing the size of these particles to the previously reported analogs by our group, there is a substantial difference in size.[93] For example, non-PEGylated, DBCO-core dendrimers of generations 6, 7, and 8, had hydrodynamic diameters of 3.21 nm, 4.50 nm, and 5.96 nm, respectively. We imagine the reason for these differences in data is likely due to intermolecular PEG chain entanglement in our samples which causes the formation of particle aggregates. The determined size of these particle aggregates is quite possibly many PEGylated dendrimers entangled with one another, which increases their overall hydrodynamic diameter. This entanglement is most likely random which explains why no general trends are present in the data. As a result, we cannot give conclusive data regarding the hydrodynamic diameter, or size, of each dendrimer individually.

We then used MALDI-MS to get an approximate molecular weight of each dendrimer and the results are summarized below in Table 3.2. It can be understood that each dendrimer has approximately, or exactly, the m/z ratio that was expected. Although some samples resulted in a m/z ratio somewhat under the expected value, which might suggest they are not fully PEGylated, we are still confident that full PEGylation did in fact occur from our ^{1}H NMR analysis. This discrepancy in data is due to inconsistent ionization of the sample, which is often observed with polydisperse samples. Preferential ionization can sometimes be biased toward smaller/lighter analytes as they tend to “fly” easier than larger/heavier ones. Furthermore, inconsistent ionization can occur from some of the of MALDI parameters used such as sample concentration, laser intensity, or number of images captured, if not kept identical between runs.

Table 3.2: Summary of MALDI-MS data for synthesized PEGylated dendrimers. All m/z values are approximate due to the dispersity of the PEG chains which give a range of m/z values on the spectrum.

Dendrimer	m/z calculated	m/z found
DBCO-G3-$(PEG\text{-}350)_8$	$[M]^+$ ~ 4.0 kDa	$[M]^+$ ~ 3.9 kDa
DBCO-G3-$(PEG\text{-}1000)_8$	$[M]^+$ ~ 9.0 kDa	$[M]^+$ ~ 7.8 kDa
DBCO-G3-$(PEG\text{-}2000)_8$	$[M]^+$ ~ 17.4 kDa	$[M]^+$ ~ 17.0 kDa
DBCO-G4-$(PEG\text{-}350)_{16}$	$[M]^+$ ~ 8.1 kDa $[M]^{2+}$ ~ 4.1 kDa	$[M]^+$ ~ 7.2 kDa $[M]^{2+}$ ~ 3.8 kDa
DBCO-G4-$(PEG\text{-}1000)_{16}$	$[M]^+$ ~ 16.0 kDa $[M]^{2+}$ ~ 8.0 kDa	$[M]^+$ ~ 14.0 kDa $[M]^{2+}$ ~ 7.9 kDa
DBCO-G4-$(PEG\text{-}2000)_{16}$	$[M]^+$ ~ 35.3 kDa $[M]^{2+}$ ~ 18.0 kDa	$[M]^+$ not found $[M]^{2+}$ ~ 17.7 kDa
DBCO-G5-$(PEG\text{-}350)_{32}$	$[M]^+$ ~ 18.0 kDa $[M]^{2+}$ ~ 9.0 kDa	$[M]^+$ not found $[M]^{2+}$ ~ 9.8 kDa
DBCO-G5-$(PEG\text{-}1000)_{32}$	$[M]^+$ ~ 39.0 kDa $[M]^{2+}$ ~ 18.5 kDa	$[M]^+$ ~ 41.0 kDa $[M]^{2+}$ ~ 19.5 kDa
DBCO-G5-$(PEG\text{-}2000)_{32}$	$[M]^+$ ~ 71.0 kDa $[M]^{2+}$ ~ 30.5 kDa	$[M]^+$ not found $[M]^{2+}$ not found

We were unable to observe a signal for DBCO-G5-$(PEG\text{-}2000)_{32}$. Although we are confident that this product was made, a signal was likely not observed because larger macromolecules typically do not "fly" or ionize well. Despite trying different sample preparation, and altering various MALDI parameters such as laser intensity, and number of images captured, we could not get even the slightest signal. Since the PEG chains on the periphery of the dendrimer are not monodisperse, this makes it even more difficult to observe a signal due to the smaller abundance of each m/z ratio found. In a monodisperse sample, 100% of the sample would be found

at a single m/z ratio, however, with a polydisperse sample, the intensity of the signal at each m/z ratio gets much smaller as it now represents a much smaller fraction of the overall polymer. For example, if a monodisperse dendrimer has a sample intensity of 100, the signal intensity of 100 will be divided among the many new m/z ratios where the polymer can be observed if it develops any degree of dispersity. If the new intensity is now 50%, or even 99% less intense than the already weakly intense monodisperse signal, it will be very difficult to differentiate this over any background noise. Overall, the MALDI-TOF MS data further corroborates the ^{1}H NMR and FTIR data presented in the previous section that showed each dendrimer was fully PEGylated.

3.6 Conclusion

A convergent synthesis approach was used to prepare the library of DBCO core, PEGylated dendrimers. Aside from using a convergent strategy, the key differences in this approach were the identity of the core protecting group, and its removal before PEGylation. This first involved preparing G3 DBCO-core dendrimers with an activated periphery which then underwent PEGylation with amine-terminated PEG chains. Following this, a series of amine core, azide periphery G1 and G2 dendrons were prepared. These were then clicked with the G3 DBCO-core PEGylated dendrimers using SPAAC chemistry to make the G4 and G5 analogs. Finally, the free amine at the core of the G4 and G5 dendrimers was coupled with a DBCO-NHS ester to give each of the dendrimers a DBCO core. All dendrimers were then further characterized using MALDI-MS and DLS to better understand their magnitude, however, DLS gave inconclusive results. This change in synthetic strategy proved to be a better alternative than the divergent strategy that was explored previously to construct the overall dendrimer library.

3.7 Experimental

General

All reagents and solvents were obtained from commercial sources and were used without further purification. DBCO derivatives were previously synthesized by Dr. Stuart McNelles. All anhydrous solvents were dried using 3 or 4 Å molecular sieves overnight prior to use. Flash chromatography was performed using an IntelliFlash 280 system from Analogix and monitored using a variable wavelength detector. Columns were prepared using Sorbtech EZ Flash Column cartridges with 25 – 40 μm silica purchased from Silicycle. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 600 MHz and 150 MHz spectrometers respectively. All chemical shifts are reported in ppm. FTIR measurements were recorded on a Nicolet 6700 FTIR from Thermo Scientific. ESI-MS spectra were obtained using a Waters-Micromass Quattro Ultima Electrospray Ionization Mass Spectrometer, or an Agilent G1969 Time-of-Flight Mass Spectrometer. MALDI-TOF MS measurements were measured on an UltrafleXtreme (Bruker Daltonics, MA, USA) in reflectron positive ion mode. Sample preparation is as follows: 1 mg of dithranol matrix was dissolved in 100 μL of THF and centrifuged for 1 min. Separately, an approximate 0.5 mg/mL solution of dendrimer was prepared in DCM. 10 μL of the dendrimer solution was mixed with 10 μL of the matrix solution. 1 μL of this solution was spotted onto the MALDI target plate and left to air dry until crystalized. DLS measurements were taken by Matthew Campea on a NanoBrook 90Plus PALS from Brookhaven.

General Synthetic Procedures

General procedure 1: G3 Dendrimer PEGylation - *Modified from literature procedures*[88]

A round bottom flask was equipped with a magnetic stir bar and charged with 1 eq. of DBCO-G3-(imidazole)$_8$, 1.5 eq./imidazole of mPEG-NH_2, 2 eq./imidazole of TEA, and DCM. The reaction

mixture was left stirring overnight at room temperature. The next day, the solvent was removed by rotary evaporation and the crude material was dissolved in ~2 mL of deionized water and purified by dialysis overnight using either 3.5 kDa or 12 kDa MWCO dialysis tubing. Dialysis water was changed twice over the span of a 24 h period and the solution was then lyophilized overnight to afford the product.

General Procedure 2: G4 & G5 Dendrimer Assembly

A round bottom flask was equipped with a magnetic stir bar and charged with 1 eq. of NH_2-Gx-$(N_3)_y$, and DCM. A solution of DBCO-G3-$(PEG)_8$ in DCM was added to the mixture in 100 µL aliquots. After every 100 µL aliquot was added, the reaction was left to stir at room temperature for 20 minutes and an FTIR measurement was taken to observe for disappearance of the azide peak at ~ 2100 cm^{-1}. If residual azide was still present, another 100 µL aliquot of DBCO-G3-$(PEG)_8$ in DCM was added, left to stir for 20 minutes, and then monitored again on FTIR. This process was repeated until complete disappearance of the azide peak was observed at which point the reaction was deemed finished. The solvent was removed by rotary evaporation and the mixture was dissolved in ~1 mL of deionized water and purified by dialysis using either 12 kDa or 100 kDa MWCO dialysis tubing. Dendrimers dialyzed with 12 kDa tubing were dialyzed overnight and water was changed twice. Dendrimers dialyzed with 100 kDa tubing were dialyzed for ~12 hours and water was changed once. After dialysis the solution was lyophilized overnight to afford the product.

General Procedure 3: Addition of DBCO Core (G4 & G5) – *Modified from literature procedures*[93]

A round bottom flask was equipped with a magnetic stir bar and charged with 1 eq. of NH_2-Gx-$(PEG)_y$, DCM, 10 eq. of DBCO-NHS and 12 eq. of TEA. The reaction mixture was left stirring at

room temperature overnight. The next day the solvent was concentrated by rotary evaporation and the crude material was directly purified using a 12 g Sorbtech EZ Flash column with a gradient of 1-2% MeOH in DCM to elute all small molecules (monitored at 254 nm), followed by isocratic elution of 20% MeOH in DCM until the dendrimer was fully eluted (monitored at 205 nm). Fractions containing dendrimer were collected, concentrated by rotary evaporation and run through a neutral alumina plug so that any residual DBCO-COOH would be retained. The plug was flushed with 100% MeOH to elute the dendrimer at which point it was concentrated by rotary evaporation, dried under vacuum, and then lyophilized overnight to afford the product.

Synthetic Procedures

NH_2-G3-$(OH)_8$ (27) - *Prepared according to literature procedures*[88]

A round bottom flask was equipped with a magnetic stir bar and charged with Cbz-G3-$(OH)_8$ (1.00 g, 0.97 mmol), $Pd(OH)_2/C$ (100 mg, 10 wt%), and a solution of 1:1 DCM:MeOH (10 mL). The reaction vessel was purged under vacuum and backfilled with hydrogen gas three times while stirring, and was then left stirring at room temperature overnight under hydrogen gas. The next day the reaction was filtered over celite, concentrated by rotary evaporation, and then dried under vacuum overnight to afford the product as a white crystalline solid. (840 mg, 97%).

^{1}H NMR (600 MHz; MeOD): δ 4.30-4.22 (m, 12H), 3.69-3.67 (m, 8H), 3.60-3.58 (m, 8H), 3.23 (t, J = 7.1 Hz, 2H), 2.85 (t, J = 7.4 Hz, 2H), 1.61-1.58 (m, 4H), 1.30 (s, 9H), 1.14 (s, 12H).

MS (ESI$^+$) *m/z* calc'd for $C_{39}H_{68}N_2O_{21}$ $[M+H]^+$ = 901.44, found $[M+H]^+$ 901.4.

DBCO-G3-$(OH)_8$ (28) - *Prepared according to literature procedures*[88]

A round bottom flask was equipped with a magnetic stir bar and charged with NH_2-G3-$(OH)_8$ (230 mg, 0.26 mmol), and MeOH (2 mL). To this, DBCO-NHS (98 mg, 0.24 mmol) dissolved in DCM (1 mL), was added, followed by TEA (53 µL, 0.38 mmol). The reaction mixture was stirred at

room temperature for 1.5 h in which point the solvent was removed by rotary evaporation. The crude residue was dissolved in 2 mL of 1:1 DMSO:H_2O and purified by reverse phase flash chromatography with 5-100% MeCN in H_2O and monitored at 205 nm. Fractions containing product were combined, and MeCN was removed by rotary evaporation. The remaining water was lyophilized over two nights to give the product as a fluffy white powder. (233 mg, 81%).

^{1}H NMR (600 MHz; MeOD): δ 7.65 (d, *J* = 7.4 Hz, 1H), 7.61-7.59 (m, 1H), 7.48-7.45 (m, 3H), 7.38-7.32 (m, 2H), 7.26-7.25 (m, 1H), 5.13 (d, *J* = 14.1 Hz, 1H), 4.30-4.22 (m, 12H), 3.71 (d, *J* = 14.1 Hz, 1H), 3.67-3.66 (m, 8H), 3.59-3.57 (m, 8H), 3.18 (t, *J* = 7.1 Hz, 2H), 3.10-3.05 (m, 2H), 2.72-2.66 (m, 1H), 2.37-2.31 (m, 1H), 2.19-2.14 (m, 1H), 2.01-1.96 (m, 1H), 1.50-1.45 (m, 2H), 1.42-1.38 (m, 2H), 1.28 (s, 9H), 1.14 (s, 12H).

MS (ESI$^+$) *m/z* calc'd for $C_{58}H_{81}N_3O_{23}$ $[M+Na]^+$ = 1210.52, found $[M+H]^+$ 1210.5.

DBCO-G3-(imidazole)$_8$ (29) – *Modified from literature procedures*[88]

A round bottom flask was equipped with a magnetic stir bar and charged with DBCO-G3-$(OH)_8$ (230 mg, 0.19 mmol) and dry MeCN (2 mL). To this, CDI (1.255 g, 7.74 mmol) dissolved in dry MeCN (8 mL), was added. The reaction mixture was stirred overnight at room temperature at which point white solids precipitated into solution. The next day the mixture was cooled in an ice bath and the precipitated material was collected by vacuum filtration, then washed with a 50 mL solution of 1:1 ether:hexanes. The product was then dried under vacuum to afford a cream coloured powder. (268 mg, 71%).

^{1}H NMR (600 MHz; DMSO-*d*6): δ 8.24 (s, 8H), 7.73 (t, *J* = 5.3 Hz, 1H), 7.68-7.61 (m, 2H), 7.55 (s, 8H), 7.49-7.42 (m, 2H), 7.38-7.28 (m, 3H), 7.03 (s, 8H), 5.01 (d, *J* = 14.1 Hz, 1H), 4.59-4.54 (m, 16H), 4.21 (s, 8H), 4.08-4.01 (m, 4H), 3.60 (d, *J* = 14.0 Hz, 1H), 2.98-2.88 (m, 4H), 2.59-2.55

(m, 1H), 2.24-2.18 (m, 1H), 1.99-1.94 (m, 1H), 1.79-1.74 (m 1H), 1.32-1.24 (m, 16H), 1.10 (s, 9H).

MS (ESI$^+$) *m/z* calc'd for $C_{90}H_{97}N_{19}O_{31}$ $[M+Na]^+$ = 1962.65, found $[M+H]^+$ 1962.7.

DBCO-G3-(PEG-350)$_8$ (30a)

Using general procedure 1, DBCO-G3-(imidazole)$_8$ (80 mg, 0.04 mmol), PEG-350-NH_2 (190 mg, 0.50 mmol), and TEA (105 µL, 0.74 mmol) were dissolved in DCM (2 mL). The mixture was purified using 3.5 kDa MWCO dialysis tubing and the product was afforded as a dark yellow oil. (131 mg, 71%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.65 (d, *J* = 5.3 Hz, 1H), 7.52-7.49 (m, 1H), 7.39-7.30 (m, 5H), 7.16-7.10 (m, 1H), 6.75 (s, 1H), 6.60 (s, 1H), 5.54 (d, *J* = 180.6 Hz, 8H), 5.13 (d, *J* = 13.9 Hz, 1H), 4.25-4.12 (m, 28H), 3.75-3.53 (m, 240H), 3.37 (s, 24H), 3.32 (s, 16H), 3.20-3.08 (m, 4H), 2.78-2.70 (m, 1H), 2.45-2.35 (m, 1H), 2.22-2.16 (m, 1H), 2.04-1.99 (m, 1H), 1.25-1.08 (m, 25H).

DBCO-G3-(PEG-1000)$_8$ (30b)

Using general procedure 1, DBCO-G3-(imidazole)$_8$ (30 mg, 0.016 mmol), PEG-1000-NH_2 (186 mg, 0.19 mmol), and TEA (39 µL, 0.28 mmol) were dissolved in DCM (2 mL). The mixture was purified using 12 kDa MWCO dialysis tubing and the product was afforded as a fluffy white powder. (75 mg, 52%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.66 (d, *J* = 5.3 Hz, 1H), 7.49-7.47 (m, 1H), 7.40-7.30 (m, 5H), 7.17-7.11 (m, 1H), 6.88 (s, 1H), 6.78 (s, 1H), 5.50 (d, *J* = 120.6 Hz, 8H), 5.13 (d, *J* = 13.9 Hz, 1H), 4.26-4.12 (m, 28H), 3.77-3.53 (m, 720H), 3.38 (s, 24H), 3.33 (s, 16H), 3.22-3.10 (m, 4H), 2.78-2.70 (m, 1H), 2.45-2.35 (m, 1H), 2.22-2.16 (m, 1H), 2.04-1.99 (m, 1H), 1.31-1.09 (m, 25H).

DBCO-G3-(PEG-2000)$_8$ (30c)

Using general procedure 1, DBCO-G3-(imidazole)$_8$ (33 mg, 0.017 mmol), PEG-2000-NH_2 (411 mg, 0.20 mmol), and TEA (43 µL, 0.31 mmol) were dissolved in DCM (2 mL). The mixture was purified using 12 kDa MWCO dialysis tubing and the product was afforded as a fluffy white powder. (190 mg, 64%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.66-7.64 (m, 1H), 7.52-7.49 (m, 1H), 7.39-7.30 (m, 5H), 7.16-7.10 (m, 1H), 6.88 (s, 1H), 6.77 (s, 1H), 5.68-5.43 (m, 8H), 5.13 (d, *J* = 13.9 Hz, 1H), 4.30-4.11 (m, 28H), 3.76-3.51 (m, 1520H), 3.38 (s, 24H), 3.32 (s, 16H), 3.20-3.09 (m, 4H), 2.78-2.70 (m, 1H), 2.45-2.35 (m, 1H), 2.22-2.16 (m, 1H), 2.04-1.99 (m, 1H), 1.31-1.13 (m, 25H).

Boc-G1-(acet)$_1$ (31)

A round bottom flask was equipped with a magnetic stir bar and charged with Bis-MPA-acetonide (1.33 g, 7.65 mmol), EDC (733 mg, 3.82 mmol) and DCM (15 mL). This was left stirring at room temperature for 30 min at which point *N*-Boc-1,4-butanediamine (360 mg, 1.91 mmol) and TEA (800 µL, 5.74 mmol) were added. The mixture was left stirring overnight. The following day the reaction mixture was quenched with 500 µL of water and stirred for 1 hour. The mixture was then diluted with 40 mL of DCM and washed with 1M H_3PO_4 (3 x 40 mL), 10% Na_2CO_3 (3 x 40 mL), and brine (1 x 40 mL). The organic layer was dried with $MgSO_4$, filtered, and then concentrated by rotary evaporation. The crude material was purified by flash chromatography using a 40 g silica Sorbtech EZ Flash column with 10-60% acetone in hexanes (loaded in DCM) and monitored at 205 nm. Fractions containing product were combined, concentrated by rotary evaporation and dried under vacuum to afford the product as a clear oil. (607 mg, 92%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.09 (s, 1H), 4.58 (s, 1H), 3.90 (d, *J* = 12.4 Hz, 2H) 3.76 (d, *J* = 12.4 Hz, 2H), 3.32 (q, *J* = 6.3 Hz, 2H), 3.14 (d, *J* = 5.6 Hz, 2H), 1.59-1.53 (m, 4H), 1.47 (s, 3H), 1.40-1.39 (m, 12H), 1.00 (s, 3H).

MS (ESI$^+$) *m/z* calc'd for $C_{17}H_{32}N_2O_5$ $[M+H]^+$ = 345.23, found $[M+H]^+$ 345.2.

Boc-G1-(OH)$_2$ (32)

A round bottom flask was equipped with a magnetic stir bar and charged with Boc-G1-(acet)$_1$ (500 mg, 1.45 mmol), Dowex® (750 mg), and MeOH (50 mL). The reaction was stirred at room temperature for 4 hours. The mixture was vacuum filtered and the solution was concentrated by rotary evaporation, then dried under vacuum to afford the product as a clear oil. (439 mg, 99%).

^{1}H NMR (600 MHz; MeOD): δ 3.63 (q, *J* = 13.5 Hz, 4H), 3.22 (t, *J* = 6.7 Hz, 2H), 3.04 (t, *J* = 6.7 Hz, 2H), 1.53-1.43 (m, 13H), 1.11 (s, 3H).

MS (ESI$^+$) *m/z* calc'd for $C_{14}H_{28}N_2O_5$ $[M+H]^+$ = 305.20, found $[M+H]^+$ 305.2.

Boc-G2-(acet)$_2$ (33)

A round bottom flask was equipped with a magnetic stir bar and charged with Bis-MPA-acetonide (686 mg, 3.94 mmol), EDC (378 mg, 1.97 mmol), and DCM (2 mL). This was left stirring at room temperature for 30 min. Separately, Boc-G1-(OH)$_2$ (150 mg, 0.49 mmol), and DMAP (30 mg, 0.25 mmol), were dissolved in pyridine (1.3 mL) and were then added to the mixture followed by TEA (293 μL, 1.97 mmol). The reaction mixture was left stirring overnight at which point it was quenched with 350 μL of water and stirred for 1 hour. The mixture was then diluted with 40 mL of DCM and washed with 1M H_3PO_4 (3 x 40 mL), 10% Na_2CO_3 (3 x 40 mL), and brine (1 x 40 mL). The organic layer was dried with $MgSO_4$, filtered, and then concentrated by rotary evaporation. The crude material was purified by flash chromatography using a 12 g silica Sorbtech EZ Flash column with 10-60% acetone in hexanes (loaded in DCM) and monitored at 205 nm.

Fractions containing product were combined, concentrated by rotary evaporation and dried under vacuum to afford the product as a clear oil. (290 mg, 95%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 6.53 (s, 1H), 4.67 (s, 1H), 4.36 (d, *J* = 11.2 Hz, 2H), 4.29 (d, *J* = 11.2 Hz, 2H), 4.17 (d, *J* = 11.9 Hz, 4H), 3.66 (d, *J* = 11.9 Hz, 4H), 3.26 (q, *J* = 6.3 Hz, 2H), 3.13-3.10 (m, 2H), 1.53-1.49 (m, 4H), 1.44 (m, 15H), 1.37 (s, 6H), 1.28 (s, 3H), 1.12 (s, 6H).

MS (ESI$^+$) *m/z* calc'd for $C_{30}H_{52}N_2O_{11}$ $[M+Na]^+$ = 639.35, found $[M+Na]^+$ 639.3.

Boc-G2-(OH)$_4$ (34)

A round bottom flask was equipped with a magnetic stir bar and charged with Boc-G2-(acet)$_2$ (280 mg, 0.45 mmol), Dowex® (420 mg), and MeOH (15 mL). The reaction was stirred at room temperature for 4 hours. The mixture was vacuum filtered and the solution was concentrated by rotary evaporation, then dried under vacuum to afford the product as a clear oil. (241 mg, 99%).

^{1}H NMR (600 MHz; MeOD): δ 4.26 (q, *J* = 9.8 Hz, 4H), 3.69 (d, *J* = 10.9 Hz, 4H), 3.60 (d, *J* = 10.9 Hz, 4H), 3.21 (q, *J* = 6.3 Hz, 2H), 3.05 (t, *J* = 6.8 Hz, 2H), 1.55-1.43 (m, 13H), 1.27 (s, 3H), 1.15 (s, 6H).

MS (ESI$^+$) *m/z* calc'd for $C_{24}H_{44}N_2O_{11}$ $[M+H]^+$ = 537.29, found $[M+H]^+$ 537.2.

Boc-G1-(N$_3$)$_2$ (35)

An oven dried round bottom flask was equipped with a magnetic stir bar, purged under vacuum and backfilled with $Ar_{(g)}$ three times before addition of **14** (225 mg, 1.31 mmol), NMI (370 µL, 4.60 mmol), dry MeCN (1 mL), and TCFH (369 mg, 1.31 mmol). This was left to stir for 10 min at room temperature at which point a solution of Boc-G1-(OH)$_2$ (100 mg, 0.33 mmol) in 500 µL of dry MeCN was added. The mixture was left stirring at room temperature for 30 min at which point it was quenched with 100 µL of water and stirred for another 5 min. The reaction mixture was then diluted with 40 mL of EtOAc and washed with water (3 x 60 mL), 1M H_3PO_4 (3 x 60

mL), 10% Na_2CO_3 (3 x 60 mL), and brine (1 x 60 mL). The organic layers were then dried with $MgSO_4$, filtered, and concentrated under rotary evaporation. The crude material was then purified using a 12 g silica Sorbtech EZ Flash column with 5-60% acetone in hexanes (loaded in DCM), and monitored at 205 nm. Fractions containing product were combined, concentrated by rotary evaporation and dried under vacuum to afford the product as a yellow oil. (142 mg, 71%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 6.20 (s, 1H), 4.63 (s, 1H), 4.23-4.19 (m, 4H), 3.30-3.26 (m, 6H), 3.13 (d, *J* = 5.9 Hz, 2H), 1.60-1.56 (m, 4H), 1.54-1.49 (m, 8H), 1.44 (s, 9H), 1.25 (s, 3H), 1.18 (s, 12H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 176.8, 172.1, 79.3, 66.4, 51.7, 46.4, 42.2, 39.9, 39.4, 37.5, 28.4, 27.7, 26.5, 25.1, 24.6, 18.0.

HRMS (ESI^+) *m/z* calc'd for $C_{28}H_{50}N_8O_7$ $[M+H]^+$ = 611.3875, found $[M+H]^+$ 611.3871.

NH_2-G1-(N_3)$_2$ (36)

A round bottom flask was equipped with a magnetic stir bar and charged with Boc-G1-(N_3)$_2$ (43 mg, 0.07 mmol) and DCM (2 mL). TFA (1 mL) was added dropwise and the reaction was stirred at room temperature for 1 hour at which point it was diluted with 10 mL of DCM and brought to pH ~14 by adding saturated KOH dropwise. The aqueous layer was extracted with DCM (6 x 20 mL) and the organic layers were washed with brine (1 x 20 mL), dried with $MgSO_4$, filtered, then concentrated under rotary evaporation. The product was dried under vacuum to afford a yellow oil. (20 mg, 56%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 6.68 (s, 1H), 4.20 (q, *J* = 9.2 Hz, 4H), 3.28-3.25 (m, 6H), 2.74 (t, *J* = 6.2 Hz, 2H), 1.59-1.49 (m, 12H), 1.24 (s, 3H), 1.18 (s, 12H),

^{13}C NMR (150 MHz; $CDCl_3$): δ 176.8, 172.0, 66.3, 51.6, 46.3, 42.2, 39.6, 37.5, 30.6, 27.0, 25.1, 24.6, 18.0.

HRMS (ESI$^+$) *m/z* calc'd for $C_{23}H_{42}N_8O_5$ $[M+H]^+$ = 511.3351, found $[M+H]^+$ 511.3361.

Boc-G2-$(N_3)_4$ (37)

An oven dried round bottom flask was equipped with a magnetic stir bar, purged under vacuum and backfilled with $Ar_{(g)}$ three times before addition of **14** (498 mg, 2.91 mmol), NMI (811 µL, 10.2 mmol), dry MeCN (1 mL), and TCFH (816 mg, 2.91 mmol). This was left to stir for 10 min at room temperature at which point a solution of Boc-G2-$(OH)_2$ (195 mg, 0.36 mmol) in 500 µL of dry MeCN was added. The mixture was left stirring at room temperature for 30 min at which point it was quenched with 200 µL of water and stirred for another 5 min. The reaction mixture was then diluted with 40 mL of EtOAc and washed with water (3 x 60 mL), 1M H_3PO_4 (3 x 60 mL), 10% Na_2CO_3 (3 x 60 mL), and brine (1 x 60 mL). The organic layers were then dried with $MgSO_4$, filtered, and concentrated under rotary evaporation. The crude material was then purified using a 12 g silica Sorbtech EZ Flash column with 5-60% acetone in hexanes (loaded in DCM), and monitored at 205 nm. Fractions containing product were combined, concentrated by rotary evaporation and dried under vacuum to afford the product as a yellow oil. (344 mg, 82%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 6.36 (s, 1H), 4.85 (s, 1H), 4.24-4.13 (m, 12H), 3.29-3.24 (m, 10H), 3.12 (d, *J* = 6.1 Hz, 2H), 1.58-1.48 (m, 20H), 1.42 (s, 9H), 1.25 (s, 9H), 1.16 (s, 24H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 176.8, 171.9, 171.4, 79.1, 67.2, 65.1, 51.7, 46.7, 42.2, 40.1, 39.6, 37.4, 28.4, 27.7, 26.7, 25.1, 24.6, 17.7.

HRMS (ESI$^+$) *m/z* calc'd for $C_{52}H_{88}N_{14}O_{15}$ $[M+H]^+$ = 1149.6626, found $[M+H]^+$ 1149.6618.

NH_2-G2-$(N_3)_4$ (38)

A round bottom flask was equipped with a magnetic stir bar and charged with Boc-G2-$(N_3)_2$ (90 mg, 0.08 mmol) and DCM (2 mL). TFA (1 mL) was added dropwise and the reaction was stirred at room temperature for 1 hour at which point it was diluted with 10 mL of DCM and brought to

pH ~14 by adding saturated KOH dropwise. The aqueous layer was extracted with DCM (6 x 20 mL) and the organic layers were washed with brine (1 x 20 mL), dried with $MgSO_4$, filtered, then concentrated under rotary evaporation. The product was dried under vacuum to afford a yellow oil. (60 mg, 73%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 6.90 (t, *J* = 5.4 Hz, 1H), 4.26-4.13 (m, 12H), 3.29-3.25 (m, 10H), 2.75 (t, *J* = 6.6 Hz, 2H), 1.61-1.49 (m, 20H), 1.26 (s, 9H), 1.17 (s, 24H).

^{13}C NMR (150 MHz; $CDCl_3$): δ 176.7, 171.9, 171.3, 67.3, 65.1, 51.6, 46.7, 42.2, 41.6, 39.8, 37.4, 27.0, 25.1, 24.6, 17.8.

HRMS (ESI^+) *m/z* calc'd for $C_{47}H_{80}N_{14}O_{13}$ $[M+H]^+$ = 1049.6102, found $[M+H]^+$ 1049.6104.

NH_2-G4-(PEG-350)$_{16}$ (39a)

Using general procedure 2, NH_2-G1-$(N_3)_2$ (9 mg, 0.018 mmol) was dissolved in 100 µL of DCM. A solution of DBCO-G3-$(PEG\text{-}350)_8$ (157 mg, 0.035 mmol) in DCM (3 mL) was added in 100 µL aliquots until complete disappearance of the azide peak was observed and reaction was deemed finished. The mixture was purified using 12 kDa MWCO dialysis tubing and the product was obtained as a sticky orange oil. (87 mg, 52%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.64-7.02 (m, 20H), 6.03 (dd, *J* = 46.8, 12.9 Hz, 2H), 5.66-5.35 (m, 16H), 4.26-4.11 (m, 64H), 3.76-3.54 (m, 480H), 3.37 (s, 48H), 3.32 (s, 32H), 3.21-3.09 (m, 12H), 1.47-1.10 (m, 85H).

NH_2-G4-(PEG-1000)$_{16}$ (39b)

Using general procedure 2, NH_2-G1-$(N_3)_2$ (5 mg, 0.010 mmol) was dissolved in 100 µL of DCM. A solution of DBCO-G3-$(PEG\text{-}1000)_8$ (184 mg, 0.020 mmol) in DCM (3 mL) was added in 100 µL aliquots until complete disappearance of the azide peak was observed and reaction was deemed

finished. The mixture was purified using 100 kDa MWCO dialysis tubing and the product was obtained as a flaky pale orange powder. (46 mg, 24%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.62-7.03 (m, 20H), 6.09-5.97 (m, 2H), 5.59-5.34 (m, 16H), 4.26-4.12 (m, 64H), 3.76-3.51 (m, 1440H), 3.37 (s, 48H), 3.32 (s, 32H), 3.25-3.14 (m, 12H), 1.30-1.12 (m, 85H).

NH_2-G4-(PEG-2000)$_{16}$ (39c)

Using general procedure 2, NH_2-G1-(N_3)$_2$ (3 mg, 0.006 mmol) was dissolved in 100 µL of DCM. A solution of DBCO-G3-(PEG-2000)$_8$ (206 mg, 0.012 mmol) in DCM (3 mL) was added in 100 µL aliquots until complete disappearance of the azide peak was observed and reaction was deemed finished. The mixture was purified using 100 kDa MWCO dialysis tubing and the product was obtained as a fluffy white powder. (31 mg, 15%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.59-7.02 (m, 20H), 6.10-5.97 (m, 2H), 5.68-5.47 (m, 16H), 4.36-4.11 (m, 64H), 3.76-3.51 (m, 3040H), 3.38 (s, 48H), 3.33 (s, 32H), 3.27-3.16 (m, 12H), 1.30-1.10 (m, 85H).

NH_2-G5-(PEG-350)$_{32}$ (40a)

Using general procedure 2, NH_2-G2-(N_3)$_4$ (15 mg, 0.014 mmol) was dissolved in 100 µL of DCM. A solution of DBCO-G3-(PEG-350)$_8$ (255 mg, 0.057 mmol) in DCM (3 mL) was added in 100 µL aliquots until complete disappearance of the azide peak was observed and reaction was deemed finished. The mixture was purified using 12 kDa MWCO dialysis tubing and the product was obtained as a sticky orange oil. (172 mg, 64%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.60-7.03 (m, 40H), 6.05-5.97 (m, 4H), 5.62-5.33 (m, 32H), 4.40-4.12 (m, 132H), 3.75-3.54 (m, 960H), 3.37 (s, 96H), 3.32 (s, 64H), 3.20-3.08 (m, 20H), 1.45-1.09 (m, 169H).

NH_2-G5-(PEG-1000)$_{32}$ (40b)

Using general procedure 2, NH_2-G2-(N_3)$_4$ (8 mg, 0.008 mmol) was dissolved in 100 µL of DCM. A solution of DBCO-G3-(PEG-1000)$_8$ (287 mg, 0.031 mmol) in DCM (3 mL) was added in 100 µL aliquots until complete disappearance of the azide peak was observed and reaction was deemed finished. The mixture was purified using 100 kDa MWCO dialysis tubing and the product was obtained as a fluffy orange oil. (62 mg, 21%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.61-7.03 (m, 40H), 6.06-5.97 (m, 4H), 5.64-5.31 (m, 32H), 4.40-4.16 (m, 132H), 3.75-3.52 (m, 2280H), 3.37 (s, 96H), 3.32 (s, 64H), 3.25-3.11 (m, 20H), 1.47-1.08 (m, 169H).

NH_2-G5-(PEG-2000)$_{32}$ (40c)

Using general procedure 2, NH_2-G2-(N_3)$_4$ (3 mg, 0.003 mmol) was dissolved in 100 µL of DCM. A solution of DBCO-G3-(PEG-1000)$_8$ (200 mg, 0.014 mmol) in DCM (3 mL) was added in 100 µL aliquots until complete disappearance of the azide peak was observed and reaction was deemed finished. The mixture was purified using 100 kDa MWCO dialysis tubing and the product was obtained as a fluffy white powder. (46 mg, 23%).

^{1}H NMR (600 MHz; $CDCl_3$): δ 7.60-7.03 (m, 40H), 6.06-5.95 (m, 4H), 5.72-5.30 (m, 32H), 4.38-4.18 (m, 132H), 3.75-3.50 (m, 6080H), 3.36 (s, 96H), 3.31 (s, 64H), 3.19-3.10 (m, 20H), 1.44-1.08 (m, 169H).

DBCO-G4-(PEG-350)$_{16}$ (41a)

Using general procedure 3, NH_2-G4-(PEG-350)$_{16}$ (83 mg, 0.009 mmol) and DBCO-NHS (35 mg, 0.088 mmol) were dissolved in 1 mL of DCM, followed by addition of TEA (34 µL, 0.25 mmol). The product was obtained as a sticky orange oil. (71 mg, 83%).

DBCO-G4-(PEG-1000)$_{16}$ (41b)

Using general procedure 3, NH_2-G4-(PEG-1000)$_{16}$ (63 mg, 0.003 mmol) and DBCO-NHS (13 mg, 0.033 mmol) were dissolved in 1 mL of DCM, followed by addition of TEA (13 µL, 0.091 mmol). The product was obtained as a sticky orange oil. (47 mg, 73%).

DBCO-G4-(PEG-2000)$_{16}$ (41c)

Using general procedure 3, NH_2-G4-(PEG-2000)$_{16}$ (41 mg, 0.001 mmol) and DBCO-NHS (5 mg, 0.011 mmol) were dissolved in 1 mL of DCM, followed by addition of TEA (2 µL, 0.014 mmol). The product was obtained as a fluffy white powder. (25 mg, 61%).

DBCO-G5-(PEG-350)$_{32}$ (42a)

Using general procedure 3, NH_2-G5-(PEG-350)$_{32}$ (71 mg, 0.004 mmol) and DBCO-NHS (15 mg, 0.038 mmol) were dissolved in 1 mL of DCM, followed by addition of TEA (6.3 µL, 0.045 mmol). The product was obtained as a sticky orange oil. (58 mg, 81%).

DBCO-G5-(PEG-1000)$_{32}$ (42b)

Using general procedure 3, NH_2-G5-(PEG-1000)$_{32}$ (37 mg, 0.001 mmol) and DBCO-NHS (4 mg, 0.010 mmol) were dissolved in 1 mL of DCM, followed by addition of TEA (1.6 µL, 0.012 mmol). The product was obtained as a sticky orange oil. (26 mg, 70%).

DBCO-G5-(PEG-2000)$_{32}$ (42c)

Using general procedure 3, NH_2-G5-(PEG-2000)$_{32}$ (46 mg, 0.0006 mmol) and DBCO-NHS (3 mg, 0.0065 mmol) were dissolved in 1 mL of DCM, followed by addition of TEA (1.1 µL, 0.008 mmol). The product was obtained as a fluffy white powder. (19 mg, 41%).

Chapter 4 – Preparation and Characterization of Dendrimer Enzyme Conjugates

4.1 Overview

In this chapter we explore the synthesis and full characterization of our dendrimer-enzyme conjugates. Due to time constraints, this was only completed for the three G3 dendrimers that were synthesized in chapter 3. We prepared these conjugates using the "grafting to" approach in which our DBCO-core dendrimers clicked with our model enzyme, α-chymotrypsin, that has been functionalized with azide residues. SPAAC chemistry allows this conjugation to occur relatively quick, and with minimal purification. To ensure that our model enzyme had been fully dendronized we characterized the dendrimer enzyme conjugates using UV-Vis spectroscopy, FTIR spectroscopy, and a quantitative NMR spectroscopic analysis. These characterization methods verified full conjugation such that all azide residues on α-chymotrypsin were fully functionalized with dendrimer, as intended.

4.2 Preparation of Dendrimer Enzyme Conjugates

Preparation of our dendronized conjugates was carried out as previously reported by our group.[93] SPAAC chemistry was utilized so that full conjugation would occur relatively easily and with minimal purification. This was achieved using azide functionalized α-chymotrypsin which was previously prepared such that there were 14 azide groups on the surface of the enzyme that could conjugate with polymer.[93] Therefore, we performed the conjugation using ~28-56 eq. of DBCO-core, PEGylated dendrimer (~2-4 eq. per azide on the functionalized α-chymotrypsin). Figure 4.1 illustrates this process. These reactions were complete in a matter of hours and

purification through dialysis was effective due to the large molecular weight difference between each polymer and its corresponding conjugate.

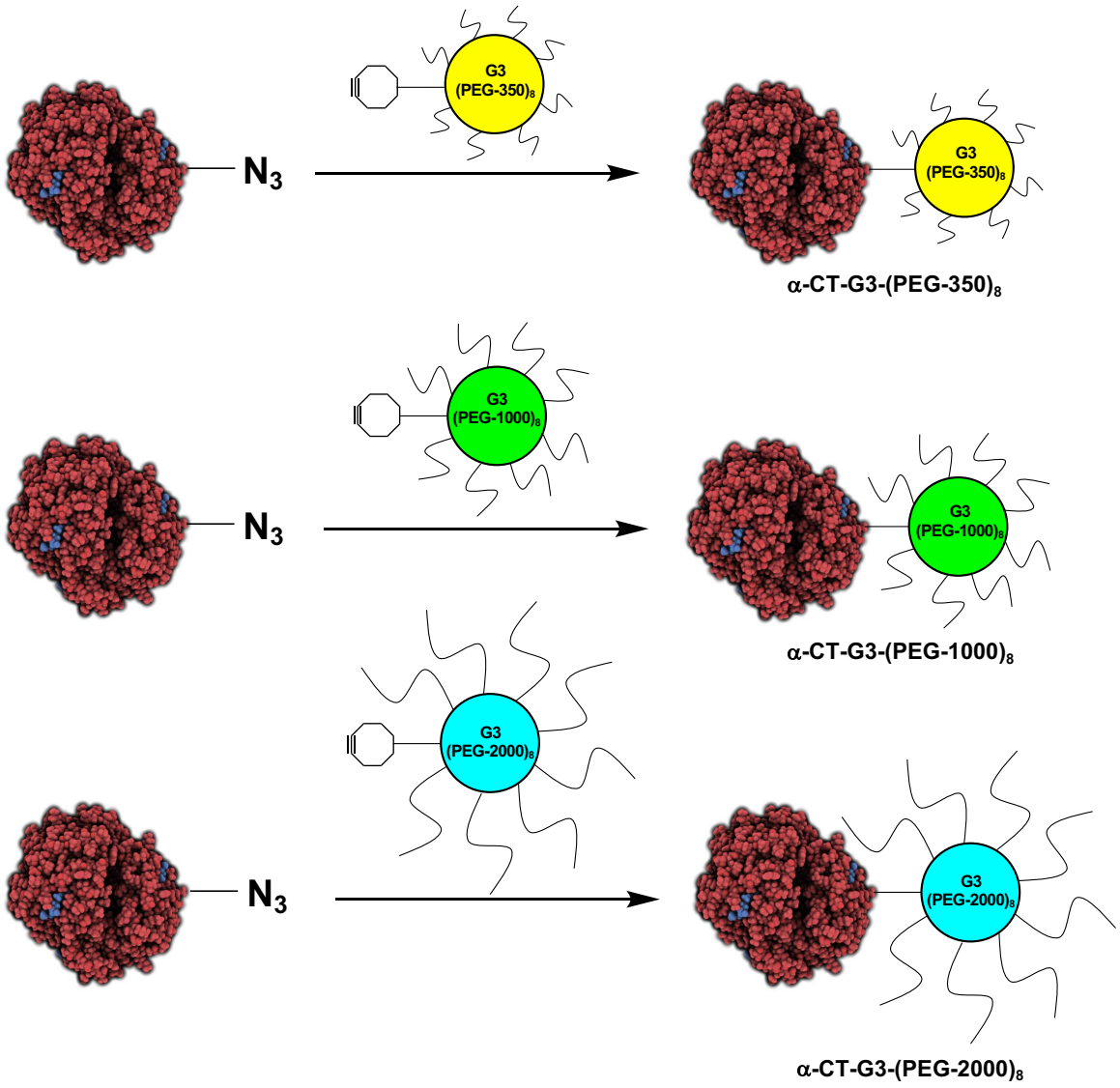


Figure 4.1: Diagrammatic representation of dendrimer-enzyme conjugation reactions. Note: Each enzyme has been functionalized with 14 dendrimers, however, only 1 is shown for simplicity. Diagram is not to scale.

4.3 Characterization of Dendrimer Enzyme Conjugates

To confirm that each azide residue on α-chymotrypsin has been conjugated with a dendrimer, we did a full characterization of each conjugate using three methods, as previously reported by our group.[93] These included a UV-Vis spectroscopic analysis, an FTIR spectroscopic analysis, and a quantitative NMR analysis.

4.3.1 UV-Vis Spectroscopy Characterization

The UV-Vis spectroscopic analysis was first completed to determine if all 14 azide residues were conjugated with our dendrimer. In this method, a stock solution of α-CT-N_3 was prepared in 0.1 M phosphate buffer and the exact mass of α-CT-N_3 in solution was calculated using the absorbance value obtained at 280 nm and the literature reported extinction coefficient of α-CT. We then did this using a DBCO-core dendrimer, however, an absorbance reading was taken at 309 nm, and we used the reported molar extinction coefficient of DBCO. Once the exact mass of dendrimer present in solution was calculated, the two solutions were mixed together. After multiple hours of reaction time an aliquot from the reaction mixture was taken and another absorbance reading was taken at 309 nm. This absorbance value was then used to calculate the mass of unreacted DBCO-core dendrimer present in solution. Since the DBCO moiety at the core of the dendrimers was being consumed upon reaction with α-CT-N_3, the corresponding absorbance reading for DBCO decreased. Once we calculated the mass of unreacted dendrimer in solution, we could then determine the mass of dendrimer that did react with α-CT-N_3. The mass of reacted dendrimer that was determined experimentally was then compared to the theoretical value that was expected to react. Since the theoretical and experimental values in all cases were quite close (experimental error < 20% for all cases), we concluded that the enzyme was fully conjugated with dendrimer. A sample calculation for this quantification is provided in the Sample Calculations

section, and Table 4.1 summarizes the findings for all three conjugates. Experimental error is likely a result of approximations made in determining the molecular weight of dendrimers, or indeterminate errors associated with sample readings and/or sample preparation.

Table 4.1: Summary of UV-Vis characterization data for dendrimer-enzyme conjugations.

Dendrimer	Theoretical Mass	Experimental Mass	% Error
DBCO-G3-(PEG-350)$_8$	16.15 mg	19.11 mg	18.3%
DBCO-G3-(PEG-1000)$_8$	15.50 mg	16.85 mg	8.7%
DBCO-G3-(PEG-2000)$_8$	11.31 mg	11.15 mg	1.4%

4.3.2 FTIR Spectroscopy Characterization

Similar to what has been reported for all SPAAC reactions in earlier chapters, we again used FTIR to observe the consumption of the azide stretch that is present on our azide functionalized α-chymotrypsin. Disappearance of this stretch at ~2100 cm^{-1} is indicative that all azide residues on our α-chymotrypsin have been reacted with a DBCO-core dendrimer. Sample spectra are shown below in Figure 4.2 of the azide functionalized α-chymotrypsin, pre-click, and a sample conjugate, post-click. Absence of the azide stretch was observed for all three conjugates post-click, clearly confirming full dendronization of each.

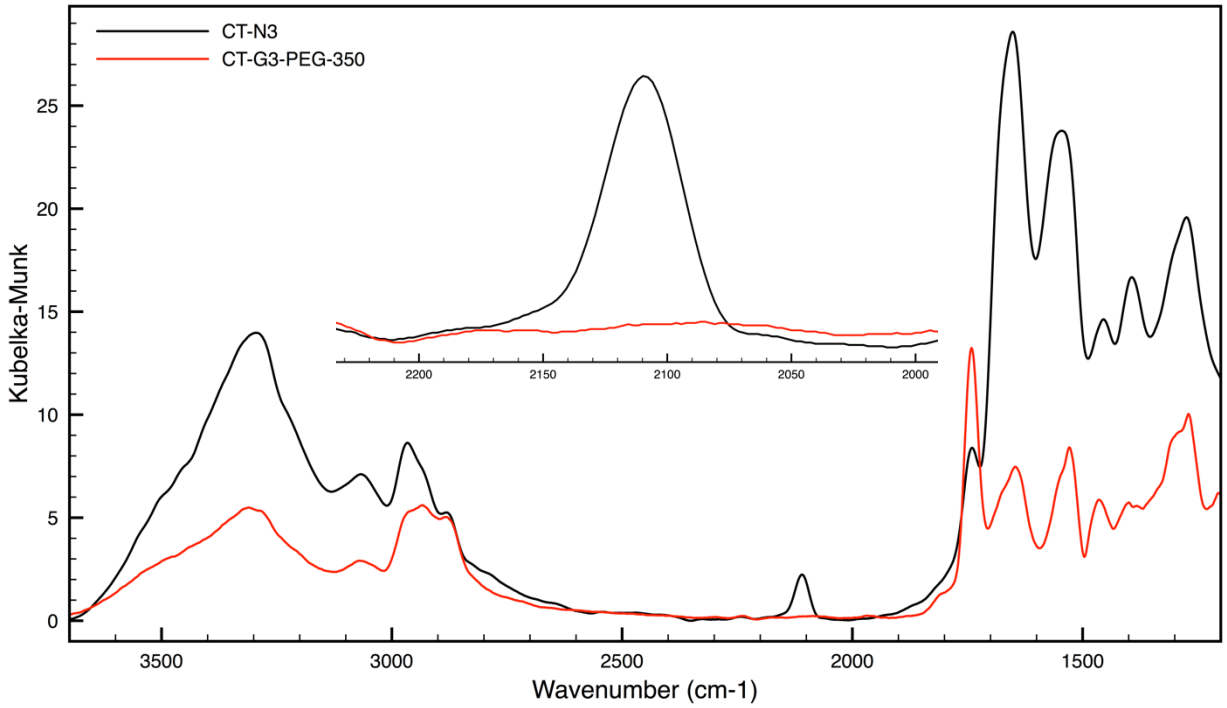


Figure 4.2. Sample FTIR spectra of α-CT-N_3 (black), and the CT-G3-PEG-350 conjugate (red) illustrating full conversion of azides.

4.3.3. Quantitative NMR Spectroscopy Characterization

Our final test for full dendronization was a quantitative NMR characterization. This was done by determining the weight fraction of the dendrimer conjugated to the enzyme against an internal standard. We first measured a known mass of the conjugate and dissolved it in D_2O. Then we added a precise amount of sodium formate (HCOONa) as an internal standard and a ^1H NMR experiment was run on the sample. A sample spectrum of the DBCO-G3-$(mPEG\text{-}2000)_8$ conjugate is shown in Figure 4.3.

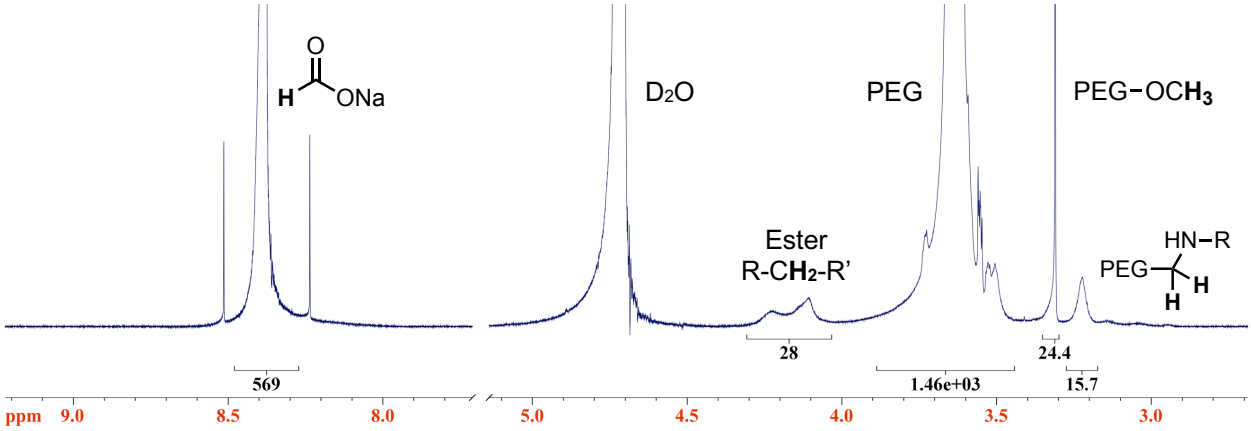


Figure 4.3. Sample ^{1}H NMR Spectrum of the DBCO-G3-(PEG-2000)$_8$ conjugate in D_2O with sodium formate as an internal standard.

In brief, the sodium formate internal standard signal (8.4 ppm) was integrated against our dendrimer signals which allowed us to calculate the number of moles of dendrimer present based on the integration ratio relative to sodium formate. Note that integrals in the spectra were set relative to the ester methylene protons of the dendrimer, as depicted in Figure 4.3 (28 ester methylene protons per dendrimer). The integration ratio, relative to the internal standard, was then used to calculate the mass of dendrimer present in the conjugate (see sample calculations in the Experimental section). Once we determined the amount of dendrimer present from NMR, we could calculate the experimental mass fraction of dendrimer in the overall conjugate. We then compared this value to the theoretical dendrimer mass fraction that was calculated under the assumption that all 14 azide residues on α-CT-N_3 were fully functionalized with exactly 14 eq. of dendrimer.

This process was completed for all of our conjugates, and the results are summarized in Table 4.2. These results corroborate the earlier findings from our UV-Vis and FTIR experiments in that all 14 azide residues on the enzyme have been fully dendronized.

Table 4.2: Summary of quantitative ^{1}H NMR characterization data for dendrimer-enzyme conjugates.

Conjugate	Mass Added	Theoretical Dendrimer Mass Fraction	Experimental Dendrimer Mass Fraction	% Error
α-CT-G3-(PEG-350)$_8$	1.7 mg	66.7%	62.7%	6.0%
α-CT-G3-(PEG-1000)$_8$	4.0 mg	81.8%	78.0%	4.6%
α-CT-G3-(PEG-2000)$_8$	1.3 mg	89.7%	87.0%	3.0%

4.4 Conclusion

In this section we synthesized our dendrimer-enzyme conjugates using our G3 dendrimers and azide functionalized α-chymotrypsin. This was achieved using SPAAC chemistry and full conjugation was achieved in just a matter of hours. Each conjugate was then isolated and carefully characterized to ensure each azide residue on α-chymotrypsin had been conjugated with a dendrimer. This was done using three methods: UV-Vis spectroscopy, FTIR spectroscopy, and a quantitative NMR analysis. All three methods of characterization confirmed full conjugation to the protein which renders each of these conjugates ready for molecular sieving studies.

4.5 Experimental

General

All experimental work in this section was completed in collaboration with Billy Deng. Azide functionalized α-chymotrypsin was previously synthesized by Dr. Stuart McNelles. ^{1}H NMR spectra were recorded on a Bruker Advance 700 MHz spectrometer in D_2O. FTIR measurements were taken on a Nicolet 6700 FTIR from Thermo Scientific. UV-Vis measurements were taken on a Cary 5000 UV-Vis NIR Spectrophotometer in single beam mode, using 700 μL quartz cuvettes with a 1 cm pathlength.

General Synthetic Procedures

General Procedure 1: Preparation of Dendrimer-Enzyme Conjugates

A vial was equipped with a magnetic stir bar and charged with approximately 28-56 eq. of DBCO-core dendrimer and 0.1 M phosphate buffer (pH ~ 8). Separately, a solution containing 1 eq. of azide functionalized α-chymotrypsin was prepared in 0.1 M phosphate buffer (pH ~ 8). The solutions were added together and stirred for around 5 hours at 4 °C. The mixture was then directly purified by dialysis using 100 kDa MWCO tubing for 24 hours and water was changed 3 times. The solution was then lyophilized overnight to afford the conjugate as a fluffy white powder.

Synthetic Procedures

α-CT-G3-(PEG-350)$_8$

Using general procedure 1, DBCO-G3-(PEG-350)$_8$ (34.2 mg, 0.0085 mmol) was dissolved in 1750 uL of 0.1 M phosphate buffer and added to a solution of α-CT-N_3 (8.1 mg, 0.00029 mmol) in 500 uL of 0.1 M phosphate buffer. The conjugate was isolated as a fluffy white powder.

α-CT-G3-(PEG-1000)$_8$

Using general procedure 1, DBCO-G3-(PEG-1000)$_8$ (67.7 mg, 0.0074 mmol) was dissolved in 1000 uL of 0.1 M phosphate buffer and added to a solution of α-CT-N_3 (3.4 mg, 0.00012 mmol) in 500 uL of 0.1 M phosphate buffer. The conjugate was isolated as a fluffy white powder.

α-CT-G3-(PEG-2000)$_8$

Using general procedure 1, DBCO-G3-(PEG-2000)$_8$ (113.2 mg, 0.0065 mmol) was dissolved in 2000 uL of 0.1 M phosphate buffer and added to a solution of α-CT-N_3 (2.9 mg, 0.00010 mmol) in 500 uL of 0.1 M phosphate buffer. The conjugate was isolated as a fluffy white powder.

4.6 Sample Calculations

Sample Calculation for Degree of Functionalization of α-CT-N_3 and DBCO-G3-$(PEG\text{-}1000)_8$ Conjugation:

$$Molar\ extinction\ coefficient\ for\ DBCO\ obtained\ from\ literature:^{109}$$

$$\varepsilon_{DBCO} = 12{,}000\ M^{-1}cm^{-1}$$

$$Extinction\ coefficient\ for\ \alpha - CT\ obtained\ from\ literature:^{110}$$

$$\varepsilon^{1\%}_{\alpha-CT} = 20.4\ \left(\frac{g}{100\ mL}\right)^{-1}(cm)^{-1}$$

$$Note: this\ coefficient\ is\ often\ reported\ as\ a\ "percent\ solution\ extinction\ coefficient"$$
$$This\ is\ because\ literature\ references\ typically\ do\ not\ provide\ molar\ extinction$$
$$coefficients\ with\ units\ of\ M^{-1}cm^{-1}.\ Instead\ they\ provide\ absorbance\ values\ for$$
$$1\%\ solutions\ which\ have\ the\ units\ of\ \left(\frac{g}{100\ mL}\right)^{-1}(cm)^{-1}\ as\ shown\ above.$$
$$When\ these\ values\ are\ applied\ as\ extinction\ coefficients\ the\ units\ of$$
$$concentration\ are\ percent\ solution.\ (ie.1\% = \frac{1\ g}{100\ mL} = \frac{10\ mg}{mL})^{111,112}$$

$$Determine\ approximate\ MW\ of\ MW\ DBCO - G3 - (PEG - 1000)_8:$$

$$Precursor\ DBCO - G3 - (imid)_8 = 1941\frac{g}{mol}$$

$$8\ imidazole\ groups\ are\ lost\ during\ PEGylation$$

$$MW\ lost = 68\frac{g}{mol} \bullet 8$$

$$MW\ lost = 544\frac{g}{mol}$$

$$8\ PEG - 1000\ chains\ are\ added\ during\ PEGylation$$
$$(MW\ of\ PEG\ confirmed\ using\ MALDI - MS)$$
$$MW\ gained = \sim 950\ \frac{g}{mol} \bullet 8$$

$$MW\ gained = \sim 7600\ \frac{g}{mol}$$

$$MW\ of\ DBCO-G3-(PEG-1000)_8 = 1941\ \frac{g}{mol} - 544\frac{g}{mol} + \sim 7600\ \frac{g}{mol}$$

$$MW\ DBCO-G3-(PEG-1000)_8 = \sim 9000\ \frac{g}{mol}$$

$Determine\ mass\ of\ dendrimer\ added\ to\ reaction$:

$$A^{309\,nm} = \varepsilon\, c\, \ell$$

$$c = \frac{A^{309\,nm}}{\varepsilon\, \ell}$$

$$c = \frac{0.44933}{(12000\ M^{-1}\cancel{cm^{-1}})(1\ \cancel{cm})}$$

$$c = 0.00003744\ M$$

$This\ represents\ the\ concenration\ of\ an\ aliquot\ taken\ from\ a\ prepared\ stock\ solution.$
$The\ dilution\ factor\ is\ 201.$

$$c = 0.00003744\ M\ \bullet 201$$

$$c\ = 0.007526244\ \frac{mmol}{mL}$$

$The\ stock\ solution\ of\ dendrimer\ was\ in\ a\ volume\ of\ 1.0\ mL$
$and\ the\ dendrimer\ MW = \sim 9000\frac{mg}{mmol}$

$$m = 0.007526244\ \frac{\cancel{mmol}}{\cancel{mL}} \bullet 1.0\ \cancel{mL} \bullet 9000\ \frac{mg}{\cancel{mmol}}$$

$$m = 67.64\ mg$$

$67.64\ mg\ of\ dendrimer\ was\ used$.

$Determine\ mass\ of\ enzyme\ used$:

$$A^{280\,nm} = \varepsilon^{1\%}\, c\, \ell$$

$$c = \frac{A^{280\,nm}}{\varepsilon^{1\%}\ \ell}\ , where\ the\ units\ of\ c = \frac{10\ mg}{mL}$$

$$c = \frac{0.04611}{\left(20.4\left(\frac{g}{100\ mL}\right)^{-1}(\cancel{cm})^{-1}\right)(1\ \cancel{cm})}$$

$$c = 0.002260294\ \frac{10\ mg}{mL}$$

$$c = 0.02260294\ \frac{mg}{mL}$$

This represents the concenration of an aliquot taken from a prepared stock solution. The dilution factor is 301.

$$c = 0.02260294\ \frac{mg}{mL} \bullet 301$$

$$c\ = 6.80348529\ \frac{mg}{mL}$$

The stock solution of dendrimer was in a volume of $0.5\ mL$.

$$m = 6.80348529\ \frac{mg}{\cancel{mL}} \bullet 0.5\ \cancel{mL}$$

$$m = 3.40\ mg$$

$3.40\ mg$ *of enzyme was used*.

Determine mass of dendrimer present in solution @ $t = 5\ h$:

$$A^{309\ nm} = \varepsilon\ c\ \ell$$

$$c = \frac{A^{309\ nm}}{\varepsilon\ \ell}$$

$$c = \frac{0.22506}{(12000\ M^{-1}\cancel{cm^{-1}})(1\ \cancel{cm})}$$

$$c = 0.000018755\ M$$

This represents the concenration of an aliquot from a prepared stock solution. The dilution factor is 201.

$$c = 0.000018755\ M\ \bullet 201$$

$$c = 0.005654632 \frac{mmol}{mL}$$

$$The\ total\ reaction\ volume = 1.5\ mL,$$
$$and\ the\ dendrimer\ MW = 9000\ mg/mmol.$$

$$m = 0.005654632 \frac{\cancel{mmol}}{\cancel{mL}} \bullet 1.5\ \cancel{mL} \bullet 9000 \frac{mg}{\cancel{mmol}}$$

$$m = 50.89\ mg$$

$$\mathbf{50.89\ mg\ of\ dendrimer\ left\ uncreacted\ in\ solution}.$$

$$Determine\ mass\ of\ dendrimer\ conjugated\ to\ enzyme:$$

$$m_{conjugated} = m_{initial} - m_{unreacted}$$

$$m_{conjugated} = 67.74\ mg - 50.89\ mg$$

$$m_{conjugated} = 16.85\ mg$$

$$\mathbf{16.85\ mg\ of\ dendrimer\ has\ been\ conjugated\ to\ the\ enzyme}.$$

$$Comapre\ to\ theoretical\ value:$$

$$\mathbf{Theoretically\ if\ all\ 14\ azide\ residues\ on\ the\ enzyme\ were\ to\ be\ conjugated\ with\ dendrimer,\ it\ would\ require\ 15.50\ mg\ (14\ eq.)\ of\ dendrimer}.$$

Sample Calculation for Degree of Functionalization of α-CT-N_3 and DBCO-G3-$(PEG\text{-}2000)_8$ Conjugation:

$$Molar\ ratio\ of\ HCOONa\ to\ dendrimer = \frac{HCOONa\ integration}{\#\ of\ H\ in\ HCOONa}$$

$$Molar\ ratio\ of\ HCOONa\ to\ dendrimer = \frac{569}{1}$$

$$There\ are\ 569\ molar\ eq.\ of\ HCOONa\ per\ dendrimer$$

$$moles\ of\ HCOONa = \frac{mass\ HCOONa}{MW\ HCOONa}$$

$$moles\ of\ HCOONa = \frac{0.0025\ g}{68.01\ \frac{g}{mol}}$$

$$moles\ of\ HCOONa = 3.676 \bullet 10^{-5}\ mol$$

Using the number of moles of HCOONa, we can find the number of moles of dendrimer that have been conjugated by dividing the moles of HCOONa by the molar eq. of HCOONa per dendrimer.

$$moles\ of\ dendrimer = \frac{3.676 \bullet 10^{-5}\ mol}{569}$$

$$moles\ of\ dendrimer = 6.460 \bullet 10^{-8}\ mol$$

$$mass\ of\ dendrimer = 6.460 \bullet 10^{-8}\ mol\ \bullet 17500\ \frac{g}{mol}$$

$$mass\ of\ dendrimer = 1.13\ mg$$

1.13 *mg of dendrimer was conjugated to the enzyme*.

The amount of conjugate added is known: 1.30 *mg*

$$Experimental\ dendrimer\ mass\ fraction = \frac{1.13\ mg}{1.30\ mg} \bullet 100$$

Experimental dendrimer mass fraction **= 87.0%.**

The theoretical mass fraction of dendrimer in the conjugate

(assuming full conjugation with 14 eq. of dendrimer) is determined as:

$$Overall\ conjugate\ MW = 273{,}000\ g/mol$$

$$MW\ of\ 14\ eq.of\ dendrimer = 245{,}000\ g/mol$$

$$Theoretical\ dendrimer\ mass\ fraction = \frac{245{,}000\ g/mol}{273{,}000\ g/mol} \bullet 100$$

Theoretical dendrimer mass fraction **= 89.7%.**

Chapter 5 – Conclusions

5.1 Conclusions

Dendrimers are a special class of polymer that have many applications due to their structural perfection, multivalent periphery, and lack of dispersity. These special characteristics of dendrimers lend themselves to a variety of applications, with a recent focus on their use in biological systems and in bioconjugation. Conjugating a polymer to a protein can instill a positive change on the overall behaviour the protein *in vivo* such as increasing the circulation half-life of the conjugate, or protecting it from an immune response. It is important however, that the polymer appended to the protein does not completely impair the protein's original function as proteins are often regulated by smaller molecules that need to interact with the protein. The molecular sieving effect was first realized in 2013 and showed that these polymer protein conjugates exhibit different interactions with various sized biomolecule substrates. While this was first realized with linear and graft polymer conjugates, recent advances using dendritic conjugates promise to be more advantageous in a clinical setting despite being more synthetically challenging.

This thesis aimed to continue exploring the possibility of the molecular sieving effect using a library of linear-dendritic hybrid polymer conjugates. While our group has recently shown that a certain generation of dendrimer is required to achieve molecular sieving, these particular dendrimers take a lot of time and effort to prepare. This prompted us to investigate whether or not we can achieve a similar sieving effect using dendrimers of lower generation that have a linear polymer appended to their periphery. These linear polymers not only help to create larger dendritic constructs in a timely manner, but the linear polymer appended also has many advantages for use in biological systems.

In Chapter 2 we investigated the synthesis of these PEGylated dendrimers using a divergent synthesis approach. We first prepared a series of bis-MPA dendrimers up to the fifth generation and then functionalized them with a small molecule azide linker. After preparing DBCO-functionalized PEG chains of varying molecular weight, we PEGylated our azide functionalized G3-G5 dendrimers using SPAAC chemistry. After each dendrimer was fully characterized to ensure full PEGylation was achieved, the core of each dendrimer needed to be deprotected to allow addition of a DBCO moiety for later conjugation to our model enzyme. We encountered an unexpected result in that the cores of most of our dendrimers were unable to undergo deprotection. We hypothesized this was due to intramolecular and/or intermolecular PEG chain entanglement which render the core inaccessible for deprotection. We attempted to resolve this issue by developing a new synthetic approach in Chapter 3.

In Chapter 3 we explored a new method for the preparation of our PEGylated dendrimer library in which we use a convergent synthesis approach. In this approach, we first prepared a series of DBCO-core, G3, PEGylated dendrimers, or "outer dendrons". We then prepared a series of amine core, G1 and G2, azide periphery dendrimers, or "inner dendrons". To construct the G4 and G5 PEGylated analogs, we used SPAAC chemistry to click our outer dendrons to our inner dendrons. Finally, the cores of the G4 and G5 derivatives were functionalized to obtain a DBCO moiety so that it could be conjugated to our model enzyme. This convergent approach proved to be a better alternative to the divergent one explored previously.

In Chapter 4, we examined the preparation and full characterization of select dendrimer enzyme conjugates. We prepared these by using SPAAC chemistry in which our DBCO-core dendrimers easily click onto azide functionalized α-chymotrypsin allowing full conjugation in a matter of hours. To ensure that our enzyme was fully conjugated with dendrimer, we fully

characterized each conjugate using 3 methods: UV-Vis, FTIR, and a quantitative NMR analysis. All three characterization methods indicated that full dendronization of the enzyme was achieved.

5.2 Recommendations for Future Work

The immediate focus of this work should be on finishing the preparation of the dendrimer enzyme conjugates using the G4 and G5 analogs prepared in Chapter 3. Once these have been prepared and fully characterized, molecular sieving studies can be performed to observe the efficacy of each of our PEGylated dendrimers in illustrating the molecular sieving effect.

While the results of these sieving studies promise to be interesting, a major limitation with this project comes with the synthesis of these large dendrimer structures that still take a lot of time and effort to make. I believe a good future direction for this project would be to determine whether or not we can observe molecular sieving using low generation (G1-G3) dendrimers that have been PEGylated with high molecular weight PEG chains. This could be achieved using identical chemistry outlined in Chapter 3 in which dendrimers can be prepared with an activated periphery, and then PEGylated with amine functionalized PEG chains. The use of high molecular weight PEG chains such as PEG 5 kDa, 10 kDa, or even 20 kDa would provide large dendritic structures, and would hopefully be similar in size to the dendrimers prepared herein. This approach would be advantageous from a synthetic standpoint as the synthesis of lower generation dendrimers and the corresponding PEGylated derivatives would be much easier and less time consuming than the methodology presented in this thesis. This would allow for the construction of dendrimers that are similar in size to those of higher generation in a fraction of the time. Furthermore, these dendrimers would still retain their globular nature and would still likely provide enough surface coverage of the enzyme such that a molecular sieving effect could be observed to some extent.

I believe that within the field of polymer chemistry, dendrimers are not entirely popular solely due to the time and effort it takes to prepare them, especially compared to other polymer architectures. If we can find a way to illustrate the molecular sieving effect using dendrimers that are simple, and quick to prepare, I believe it would bring a lot more attention to this field.

References

(1) Rasmussen, S. C. *Ambix* **2018**, *65* (4), 356–372.

(2) Peleshanko, S.; Tsukruk, V. V. *Prog. Polym. Sci.* **2008**, *33* (5), 523–580.

(3) Hawker, C. J.; Malmström, E. E.; Frank, C. W.; Kampf, J. P. *J. Am. Chem. Soc.* **1997**, *119* (41), 9903–9904.

(4) Yates, C. R.; Hayes, W. *Eur. Polym. J.* **2004**, *40* (7), 1257–1281.

(5) Fournier, D.; Hoogenboom, R.; Schubert, U. S. Clicking Polymers: *Chem. Soc. Rev.* **2007**, *36* (8), 1369–1380.

(6) Walter, M. V.; Malkoch, M. *Chem. Soc. Rev.* **2012**, *41* (13), 4593–4609.

(7) Bosman, A. W.; Janssen, H. M.; Meijer, E. W. *Chem. Rev.* **1999**, *99* (7), 1665–1688.

(8) Gitsov, I.; Wooley, K. L.; Hawker, C. J.; Ivanova, P. T.; Frechet, J. M. J. *Macromolecules* **1993**, *26* (21), 5621–5627.

(9) Jansen, J. F. G. A.; Berg, E. M. M. de B. den; Meijer, E. W. *Science* **1994**, *266* (5188), 1226–1229.

(10) Abbasi, E.; Aval, S. F.; Akbarzadeh, A.; Milani, M.; Nasrabadi, H. T.; Joo, S. W.; Hanifehpour, Y.; Nejati-Koshki, K.; Pashaei-Asl, R. *Nanoscale Res. Lett.* **2014**, *9* (1), 247.

(11) Balzani, V.; Ceroni, P.; Gestermann, S.; Kauffmann, C.; Gorka, M.; Vögtle, F. *Chem. Commun.* **2000**, *(10)*, 853–854.

(12) Longmire, M.; Choyke, P. L.; Kobayashi, H. *Curr. Top. Med. Chem.* **2008**, *8* (14), 1180–1186.

(13) Pedziwiatr-Werbicka, E.; Milowska, K.; Dzmitruk, V.; Ionov, M.; Shcharbin, D.; Bryszewska, M. *Eur. Polym. J.* **2019**, *119*, 61–73.

(14) Scott, R. W. J.; Wilson, O. M.; Crooks, R. M. *J. Phys. Chem. B* **2005**, *109* (2), 692–704.

(15) Buhleier, E.; Wehner, W.; Vogtle, F. *Synthesis* **1978**, *2*, 155-158.

(16) Tomalia, D. A.; Baker, H.; Dewald, J.; Hall, M.; Kallos, G.; Martin, S.; Roeck, J.; Ryder, J.; Smith, P. *Polym. J.* **1985**, *17* (1), 117–132.

(17) Lyu, Z.; Ding, L.; Huang, A. Y.-T.; Kao, C.-L.; Peng, L. *Mater. Today Chem.* **2019**, *13*, 34–48.

(18) de Brabander-van den Berg, E. M. M.; Meijer, E. W. *Angew. Chem. Int. Ed. Engl.* **1993**, *32* (9), 1308–1311.

(19) Kaur, D.; Jain, K.; Mehra, N. K.; Kesharwani, P.; Jain, N. K. *J. Nanoparticle Res.* **2016**, *18* (6), 146.

(20) Carlmark, A.; Hawker, C.; Hult, A.; Malkoch, M. *Chem. Soc. Rev.* **2009**, *38* (2), 352–362.

(21) Esfand, R.; Tomalia, D. A. *Drug Discov. Today* **2001**, *6* (8), 427–436.

(22) Hawker, C. J.; Frechet, J. M. J. *J. Am. Chem. Soc.* **1990**, *112* (21), 7638–7647.

(23) Hawker, C.; Fréchet, J. M. J. *J. Chem. Soc. Chem. Commun.* **1990**, No. 15, 1010–1013.

(24) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2001**, *40* (11), 2004–2021..

(25) Membreno, R.; Keinänen, O. M.; Cook, B. E.; Tully, K. M.; Fung, K. C.; Lewis, J. S.; Zeglis, B. M. *Mol. Pharm.* **2019**, *16* (5), 2259–2263.

(26) Cook, B. E.; Membreno, R.; Zeglis, B. M. *Bioconjug. Chem.* **2018**, *29* (8), 2734–2740.

(27) McNelles, S.; Knight, S.; Janzen, N.; Valliant, J.; Adronov, A. *Biomacromolecules* **2015**, *16*, 3033-3041.

(28) García-Gallego, S.; Andrén, O. C. J.; Malkoch, M. *J. Am. Chem. Soc.* **2020**, *142* (3), 1501–1509.

(29) Vieyres, A.; Lam, T.; Gillet, R.; Franc, G.; Castonguay, A.; Kakkar, A. *Chem. Commun.* **2010**, *46* (11), 1875–1877.

(30) Sadowski, L. P.; Edem, P. E.; Valliant, J. F.; Adronov, A. *Macromol. Biosci.* **2016**, *16*, 1475-1484.

(31) Antoni, P.; Robb, M. J.; Campos, L.; Montanez, M.; Hult, A.; Malmström, E.; Malkoch, M.; Hawker, C. J. *Macromolecules* **2010**, *43* (16), 6625–6631.

(32) Ornelas, C.; Ruiz Aranzaes, J.; Cloutet, E.; Alves, S.; Astruc, D. *Angew. Chem. Int. Ed.* **2007**, *46* (6), 872–877.

(33) Savithri, J. S.; Rajakumar, P. *New J. Chem.* **2018**, *42* (24), 19390–19399.

(34) Tulli, L. G.; Miranda, D.; Lee, C. C.; Sullivan, Y.; Grotzfeld, R.; Hollingworth, G.; Kneuer, R.; Karpov, A. S. *Org. Biomol. Chem.* **2019**, *17* (11), 2906–2912.

(35) McNelles, S. A.; Thoma, J. L.; Adronov, A.; Duhamel, J. *Macromolecules* **2018**, *51* (4), 1586–1590.

(36) Wang, Y.; Huang, D.; Wang, X.; Yang, F.; Shen, H.; Wu, D. *Biomater. Sci.* **2019**, *7* (8), 3238–3248.

(37) Naguib, H.; Cao, X.; Gao, H. *Macromol. Chem. Phys.* **2019**, *220* (17), 1900221.

(38) Wu, P.; Feldman, A. K.; Nugent, A. K.; Hawker, C. J.; Scheel, A.; Voit, B.; Pyun, J.; Fréchet, J. M. J.; Sharpless, K. B.; Fokin, V. V. *Angew. Chem. Int. Ed.* **2004**, *43* (30), 3928–3932.

(39) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem. Int. Ed.* **2009**, *48* (38), 6974–6998.

(40) Shi, W.; Tang, F.; Ao, J.; Yu, Q.; Liu, J.; Tang, Y.; Jiang, B.; Ren, X.; Huang, H.; Yang, W.; Huang, W. *Angew. Chem. Int. Ed.* ***2020****, 59* (45), 19940-19944.

(41) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126* (46), 15046–15047.

(42) Dommerholt, J.; Rutjes, F. P. J. T.; van Delft, F. L. *Top. Curr. Chem. Cham* **2016**, *374* (2).

(43) Chadwick, R. C.; Van Gyzen, S.; Liogier, S.; Adronov, A. *Synthesis* **2014**, *46* (05), 669-677.

(44) Turner, R. B.; Jarrett, A. D.; Goebel, P.; Mallon, B. J. *J. Am. Chem. Soc.* **1973**, *95* (3), 790–792.

(45) Ramil, C. P.; Lin, Q. *Chem. Commun.* **2013**, *49* (94), 11007–11022.

(46) Sletten, E. M.; Bertozzi, C. R. *Acc. Chem. Res.* **2011**, *44* (9), 666–676.

(47) Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130* (34), 11486–11493.

(48) McKay, C. S.; Finn, M. G. *Chem. Biol.* **2014**, *21* (9), 1075–1101.

(49) Debets, M. F.; van Berkel, S. S.; Dommerholt, J.; Dirks, A. (Ton) J.; Rutjes, F. P. J. T.; van Delft, F. L. *Acc. Chem. Res.* **2011**, *44* (9), 805–815.

(50) Borchmann, D. E.; Carberry, T. P.; Weck, M. *Macromol. Rapid Commun.* **2014**, *35* (1), 27–43.

(51) Pelegri-O'day, E.; Lin, E.; Maynard, H. *J. Am. Chem. Soc.* **2014**, *136*, 14323-14332.

(52) Romero, O.; Rivero, C. W.; Guisan, J. M.; Palomo, J. M. *PeerJ* **2013**, *1*, e27.

(53) Abuchowski, A.; van Es, T.; Palczuk, N. C.; Davis, F. F. *J. Biol. Chem.* **1977**, *252* (11), 3578-3581.

(54) Wang, X.; Yadavalli, N. S.; Laradji, A. M.; Minko, S. *Macromolecules* **2018**, *51* (14), 5039–5047.

(55) Carmali, S.; Murata, H.; Cummings, C.; Matyjaszewski, K.; Russell, A. J. *Methods in Enzymology* **2017**, *590*, 347-380.

(56) Kaupbayeva, B.; Russell, A. J. *Prog. Polym. Sci.* **2020**, *101*, 101194.

(57) Macchione, M. A.; Biglione, C.; Strumia, M. *Polymers* **2018**, *10* (5), 527.

(58) Zhang, Q.; Chen, G.; Liu, X.; Qian, Q. *Cell Res.* **2007**, *17* (2), 89–99.

(59) Kunamneni, A.; Ogaugwu, C.; Goli, D. *Enzymes in Human and Animal Nutrition* **2018**, 301-213.

(60) Vicent, M. J.; Dieudonné, L.; Carbajo, R. J.; Pineda-Lucena, A. *Expert Opin. Drug Deliv.* **2008**, *5* (5), 593–614.

(61) Pasut, G. *Polymers* **2014**, *6* (1), 160–178.

(62) Ekladious, I.; Colson, Y. L.; Grinstaff, M. W. *Nat. Rev. Drug Discov.* **2019**, *18* (4), 273–294.

(63) Wright, T. A.; Page, R. C.; Konkolewicz, D. *Polym. Chem.* **2019**, *10* (4), 434–454.

(64) Veronese, F. M.; Mero, A. *BioDrugs* **2008**, *22* (5), 315–329.

(65) Keefe, A. J.; Jiang, S. *Nat. Chem.* **2012**, *4* (1), 59–63.

(66) Abuchowski, A.; McCoy, J. R.; Palczuk, N. C.; van Es, T.; Davis, F. F. *J. Biol. Chem.* **1977**, 252, 3582–3586.

(67) Shah, S.; Prematta, T.; Adkinson, N. F.; Ishmael, F. T. J. *Clin. Pharmacol.* **2013**, *53*, 352.

(68) Schellekens, H.; Hennink, W. E.; Brinks, V. *Pharm. Res.* **2013**, *30*, 1729.

(69) Hou, S.; Trochimczyk, P.; Sun, L.; Wisniewska, A.; Kalwarczyk, T.; Zhang, X.; Wielgus-Kutrowska, B.; Bzowska, A.; Holyst, R. *Sci. Rep.* **2016**, *6* (1), 22033.

(70) Rodríguez-Martínez, J. A.; Rivera-Rivera, I.; Solá, R. J.; Griebenow, K. *Biotechnol. Lett.* **2009**, *31* (6), 883–887.

(71) Pasut, G.; Sergi, M.; Veronese, F. M. *Adv. Drug Deliv. Rev.* **2008**, *60* (1), 69–78.

(72) Kaminskas, L. M.; Boyd, B. J.; Porter, C. J. H. *Nanomedicine* **2011**, *6* (6), 1063-1084.

(73) Gillies, E. R.; Dy, E.; Fréchet, J. M. J.; Szoka, F. C. *Mol. Pharm.* **2005**, 2 (2), 129– 138.

(74) Gillies, E. R.; Fréchet, J. M. J. *Drug Discov. Today* **2005**, *10* (1), 35–43.

(75) Lee, C. C.; MacKay, J. A.; Fréchet, J. M. J.; Szoka, F. C. *Nat. Biotechnol.* **2005**, *23*, 1517-1526.

(76) Zhang, Y.; Mesa-Antunez, P.; Fortuin, L.; Andrén, O. C. J.; Malkoch, M. *Biomacromolecules* **2020**, *21* (10), 4294-4301.

(77) Feliu, N.; Walter, M. V.; Montañez, M. I.; Kunzmann, A.; Hult, A.; Nyström, A.; Malkoch, M.; Fadeel, B. *Biomaterials* **2012**, *33*, 1970−1981.

(78) Stenström, P.; Hjorth, E.; Zhang, Y.; Andreń, O. C. J.; Guette- Marquet, S.; Schultzberg, M.; Malkoch, M. *Biomacromolecules* **2017**, *18*, 4323−4330.

(79) Gitsov, I. *J. Polym. Sci. A1.* **2008**, *46*, 5295-5314.

(80) Twibanire, J. K.; Grindley, T. B. *Polymers* **2012**, *4* (1), 794–879.

(81) Gillies, E. R.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **2002**, *124* (47), 14137–14146.

(82) Yang, H.; Morris, J. J.; Lopina, S. T. *J. Colloid Interface Sci.* **2004**, *273* (1), 148–154.

(83) Gajbhiye, V.; Vijayaraj Kumar, P.; Tekade, R. K.; Jain, N. K. *Eur. J. Med. Chem.* **2009**, *44* (3), 1155–1166.

(84) Khandare, J. J.; Jayant, S.; Singh, A.; Chandna, P.; Wang, Y.; Vorsa, N.; Minko, T. *Bioconjug. Chem.* **2006**, *17* (6), 1464–1472.

(85) Ho, M. N.; Bach, L. G.; Nguyen, D. H.; Nguyen, C. H.; Nguyen, C. K.; Tran, N. Q.; Nguyen, N. V.; Thi, T. T. H. *Biopolymers* **2019**, *110* (7), e23272.

(86) Yang, Z.; Zhang, W.; Liu, J.; Shi, W. *Colloids Surf. B Biointerfaces* **2007**, *55* (2), 229–234.

(87) Laskar, P.; Somani, S.; Altwaijry, N.; Mullin, M.; Bowering, D.; Warzecha, M.; Keating, P.; Tate, R. J.; Leung, H. Y.; Dufès, C. *Nanoscale* **2018**, *10* (48), 22830–22847.

(88) McNelles, S. A.; Adronov, A. *Macromolecules* **2017**, *50* (20), 7993–8001.

(89) Zhang, C.; Pan, D.; Luo, K.; Li, N.; Guo, C.; Zheng, X.; Gu, Z. *Polym. Chem.* **2014**, *5* (18), 5227–5235.

(90) Lo Conte, M.; Robb, M. J.; Hed, Y.; Marra, A.; Malkoch, M.; Hawker, C. J.; Dondoni, A. *J. Polym. Sci. A1.* **2011**, *49* (20), 4468–4475.

(91) Ornelas, C.; Broichhagen, J.; Weck, M. *J. Am. Chem. Soc.* **2010**, *132* (11), 3923-3931.

(92) Liu, M.; Tirino, P.; Radivojevic, M.; Phillips, D. J.; Gibson, M. I.; Leroux, J.-C.; Gauthier, M. A. *Adv. Funct. Mater.* **2013**, *23* (16), 2007–2015.

(93) McNelles, S.; Marando, V.; Adronov, A. *Angew. Chem. Int. Ed.* **2019**, *58* (25), 8848-8853.

(94) Kaupbayeva, B.; Murata, H.; Lucas, A.; Matyjaszewski, K.; Minden, J. S.; Russell, A. J. *Biomacromolecules* **2019**, *20* (3), 1235–1245.

(95) Liu, M.; Johansen, P.; Zabel, F.; Leroux, J.-C.; Gauthier, M. A. *Nat. Commun.* **2014**, *5*, 1-8.

(96) Lucas, A.; Kaupbayeva, B.; Murata, H.; Cummings, C.; Russell, A. J.; Minden, J. S. *ACS Appl. Polym. Mater.* **2019**, *1* (11), 2897–2906.

(97) García-Gallego, S.; Hult, D.; Olsson, J. V.; Malkoch, M. *Angew. Chem. Int. Ed.* **2015**, *54* (8), 2416–2419.

(98) Beutner, G. L.; Young, I. S.; Davies, M. L.; Hickey, M. R.; Park, H.; Stevens, J. M.; Ye, Q. *Org. Lett.* **2018**, *20* (14), 4218–4222.

(99) Vutukuri, D, R.; Bharathi, P.; Yu, Z.; Rajasekaran, K.; Tran, M.; Thayumanavan, S. *J. Org. Chem.* **2003**, *68*, 1146-1149.

(100) Jacquemard, U.; Bénéteau, V.; Lefoix, M.; Routier, S; Mérour, J-Y.; Coudert, G. *Tetrahedron* **2004**, *60*, 10039-10047.

(101) Chen, G-H.; Pan, P.; Chen, Y.; Meng, X-B.; Li, Z-J. *Tetrahedron* **2009**, *65*, 5922-5927.

(102) Kim, J. D.; Han, G.; Zee, O. P.; Jung, Y. H. *Tetrahedron Lett.* **2003**, *44*, 733-735.

(103) Parrott, M. C.; Benhabbour, S. R.; Saab, C.; Lemon, J. A.; Parker, S.; Valliant, J. F.; Adronov, A. *J. Am. Chem. Soc.* **2009**, *131* (8), 2906–2916.

(104) Ihre, H.; Hult, A.; Fréchet, J. M. J.; Gitsov, I. *Macromolecules* **1998**, *31* (13), 4061–4068.

(105) González-Fernández, D.; Torneiro, M.; Lazzari, M. *Polymers* **2020**, *12* (6), 1269.

(106) Goswami, L. N.; Houston, Z. H.; Sarma, S. J.; Jalisatgi, S. S.; Hawthorne, M. F. *Org. Biomol. Chem.* **2013**, *11* (7), 1116–1126.

(107) Hattori, T.; Tsubone, A.; Sawama, Y.; Monguchi, Y.; Sajiki, H. *Tetrahedron* **2014**, *70* (32), 4790–4798.

(108) Behl, G.; Sikka, M.; Chhikara, A.; Chopra, M. *J. Colloid Interface Sci.* **2014**, *416*, 151–160.

(109) Liu, D.; Yang, J.; Wang, H. F.; Wang, Z.; Huang, X.; Wang, Z.; Niu, G.; Hight Walker, A. R.; Chen, X. *Anal. Chem.* **2014**, *86*, 5800–5806.

(110) Volini, M.; Tobias, P. *J. Biol. Chem.* **1969**, *244*, 5105–5109.

(111) Gill, S. C.; von Hippel, P. H. *Anal. Biochem.* **1989**, *182*, 319-326.

(112) Pace, C. N., et al. *Protein Sci.* **1995**, *4*, 2411-2423.