Biocompatible, pH-Responsive Amphiphilic Linear Dendritic Block Copolymers for Drug Delivery

by

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Abstract

Linear dendritic block copolymers are a class of dendritic nanomaterials composed of a dendron and a polymer. The block structure allows the preparation of amphiphilic macromolecules that can self-assemble into various nanostructures. Their asymmetrical molecular structure, due to the presence of a short and condensed dendron and a long linear polymeric chain, gives them unusual self-assembly properties. Furthermore, the dendron surface can be precisely functionalized giving to their self-assembled structure interesting properties that could be exploited for a variety of applications. In this thesis, a series of pH-responsive amphiphilic linear dendritic block copolymers has been synthesized, and their aqueous self-assembly examined before being evaluated for *in vitro* drug delivery. The linear dendritic block copolymers synthesis is conducted via a combination of efficient Cu(I) alkyne azide cycloaddition "click" reaction and Steglish esterification to prepare multiple generations of bis-MPA dendrons with different PEG length. The pH sensitivity of the system was brought in by functionalizing the dendron with acetonide units at the surface. LDBCs were fully characterized by various techniques including ^{1}H- ^{13}C-NMR spectroscopy, GPC and MALDI-TOF. The LDBC self-assembly was carried out by co-solvent evaporation method, and it yielded two types of nanostructures, spherical micelles and rod-like micelles, which were analysed by DLS and TEM. **G1PEG2000** has been selected for a detailed analysis of its pH-responsiveness showing swelling and shape-shifting effect of the pH on the micellar structure. Finally, the potential of the **G1PEG2000** for drug delivery was explored by encapsulating acetazolamide, a potential anticancer drug, inside micelles. The amount of acetazolamide encapsulated was quantified, and cancer cell spheroids treated with the loaded micelles. The *in vitro* study showed increased cancer cell

death when treated with the acetazolamide micelle and proved the efficiency of the developed system for drug delivery.

Résumé

Les copolymères à blocs dendritiques linéaires sont une classe de nanomatériaux dendritiques composés d'un dendron et un polymère. La structure en bloc permet la préparation de macromolécules amphiphiles qui peuvent s'auto-assembler en diverses nanostructures. Leurs structures moléculaires asymétriques leurs confèrent des propriétés exceptionnelles d'auto-assemblage en raison de la présence d'un dendron court et condensé, ainsi qu'une longue chaîne linéaire polymérique. En outre, la surface des dendrons peut être fonctionnalisée avec précision, permettant aux structures auto-assemblées d'exprimer d'intéressantes propriétés qui peuvent être exploitées pour des applications variées. Dans cette thèse, une série de copolymères à blocs amphiphiles dendritiques linéaires répondant au pH a été synthétisé, leur auto-assemblage en phase aqueuse examiné, et l'étude la livraison d'agent thérapeutique testé *in vitro*. La synthèse de copolymères à blocs dendritiques linéaires a été réalisée via une combinaison de deux réactions: cycloaddition d'azoture et d'alcyne catalysée par du Cuivre (I) « click-réaction », et l'estérification de Steglish, afin de préparer de multiples générations de dendrons bis-MPA avec différentes longueurs de PEG. La sensibilité au pH du système a été gouvernée par la fonctionnalisation des dendrons en surface, par des unités acétonides. Les copolymères à blocs dendritiques linéaires ont été complètement caractérisés par différentes techniques dont ^{1}H, ^{13}C RMN, GPC et MALDI-TOF. L'auto-assemblage de copolymères à blocs dendritiques linéaires a été conduit par un procédé de co-évaporation de solvant, et a produit deux types de nanostructures: des micelles sphériques et des micelles en forme de tige, qui ont été analysés par DLS et MET. **G1PEG2000** a été sélectionné pour une analyse détaillée de son activité vis-à-vis du pH, montrant le gonflement et le changement de forme

de la structure micellaire. Enfin, le potentiel de livraison d'agent thérapeutique de **G1PEG2000** a été exploré par l'encapsulation d'acétazolamide, un médicament potentiellement anticancéreux, à l'intérieur des micelles. La quantité de l'acétazolamide encapsulé a été quantifiée, et des sphéroïdes de cellules cancéreuses ont été traités avec des micelles chargées. L'étude *in vitro* a montré une augmentation de la mort des cellules cancéreuses, lorsqu'elles sont traitées avec les micelles contenant l'acétazolamide, ce qui démontre l'efficacité du système mis au point pour l'administration de médicaments.

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AFM	Atomic Force Microscopy
ATZ	Acetalozamide
bis-MPA	2,2-bis
$CaCO_3$	Calcium Carbonate
$CHCl_3$	Chloroform
CuAAC	Copper (I) alkyne azide cycloaddition
$CuSO_4 \cdot 5H_2O$	Copper(II) sulphate pentahydrate
CMC	Critical Micelle Concentration
CT Scan	Computed Tomography Scan
DCC	n,n'-dicylcohexylcarbodiimide
DCM	Dichloromethane
DLS	Dynamic Light Scattering
DMAP	4-dimethylaminopyridine
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DNQ	Diazonaphtoquilone
DMSO	Dimethyl Sulfoxide
EDC·HCl	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride salt
EGF	Epidermal Growth Factor
ESI	Electron Spray Ionization
GPC	Gel Permeation Chromatography
HPLC	High-Performance Liquid Chromatography
HRMS	High Resolution Mass Spectroscopy
LDBC	Linear-Dendritic Block Copolymers
MALDI-TOF	Matrix Assisted Light Desorption Ionization – Time of Flight
MeOH	Methanol

M_n	Molecular weight
MsCl	Methanesulfonyl chloride
MS	Mass Spectroscopy
M_w/M_n	Molecular Weight Distribution
MW	Molecular Weight
Na_2EDTA	Ethylenediamine tetraacetate disodium salt
NaOH	Sodium Hydroxide
Na_2SO_4	Sodium Sulfate
NEt_3	Triethyl Amine
NIR	Near Infrared
NMR	Nuclear Magnetic Resonance
PAMAM	Poly(Amidoamine)
PDI	Polydispersity Index
PEG	Poly(ethylene glycol)
PETA	Pentaethylenehexamine
PNIPAM	poly(N-isopropylacrylamide)
p-TSA	p-toluene sulfonic acid
PVDF	Polyvinylidene Fluoride
PZLL	Poly-(N-E-Carbobenzyloxy-L-Lysine)
Rh	Hydrodynamic Radius
SD	Standard Deviation
SEM	Scanning Electron Microscope
siRNA	Small Interfering Ribonucleic Acid
S.E.M	Standard Error of the Mean
TEM	Transmission Electron Microscopy
THF	Tetrahydrofuran
TMS	Tetramethylsilane
UAc_2	Uranyl Acetate
UV	Ultraviolet

Chapter 1: Introduction

1.1 - Dendrimers and dendritic nanomaterials

Dendrimers are a class of hyperbranched macromolecules first introduced by Vögtle in 1969.[1] Originally described as cascade molecules due to the iterative process to synthesize them, they were later given the name "dendrimer" from the Greek *dendron* meaning tree, which refers to the tree-like structure of their hyperbranched structure. This class of macromolecules distinguished itself from others by their globular shape, multivalent surface and monodisperse nature. These unique characteristics come from the layer-by-layer, controlled synthesis of dendrimers.[2] The core of the dendrimer is linked to multiple branched monomer giving rise to a hyperbranched structure. By increasing the number of branching iterations, the generations of the dendrimer are increased, as well as the size and weight of the dendrimer.

Two synthetic methods are generally used to prepare dendrimers: divergent and convergent, which are described in Figure 1.1. The divergent synthesis was first developed by Tomalia in 1985[3] for the synthesis of the poly(amidoamine) (PAMAM) dendrimers (Scheme 1.1). It is carried out by starting from the core and growing the dendrimer outwards by a layer-by-layer methodology. The convergent synthesis was developed 5 years later by Hawker and Fréchet,[4] where dendrons are first prepared and then attached to the core to give the final dendrimer. Both methods use an iterative synthesis, as opposed to polymerization synthesis, yielding monodisperse macromolecules and allowing easy functionalization of the surface to tune the properties of the dendrimer.

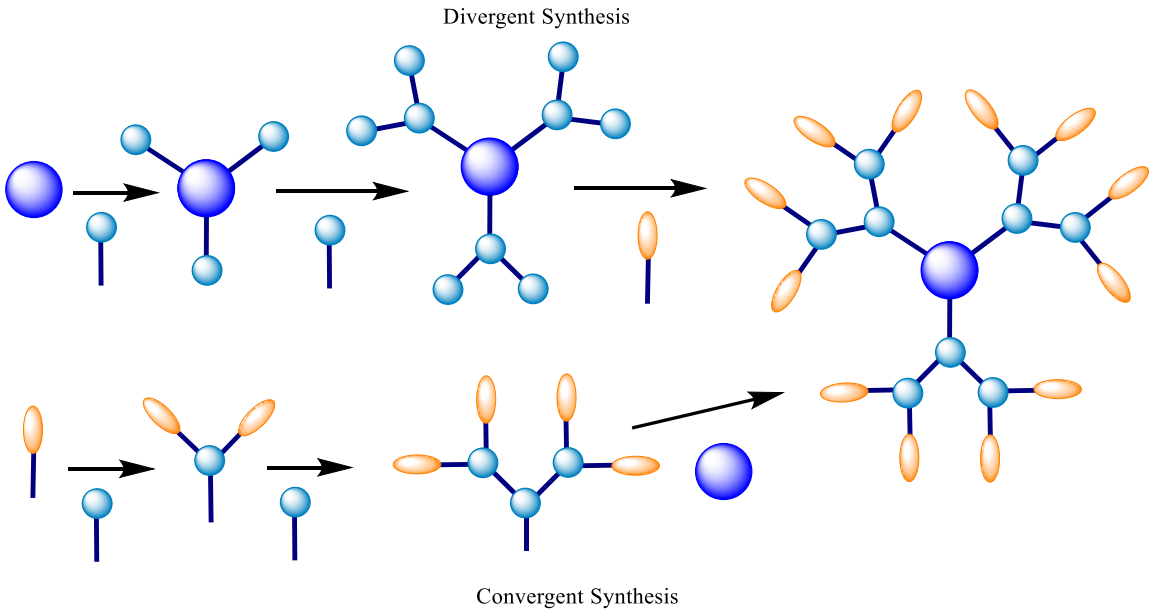
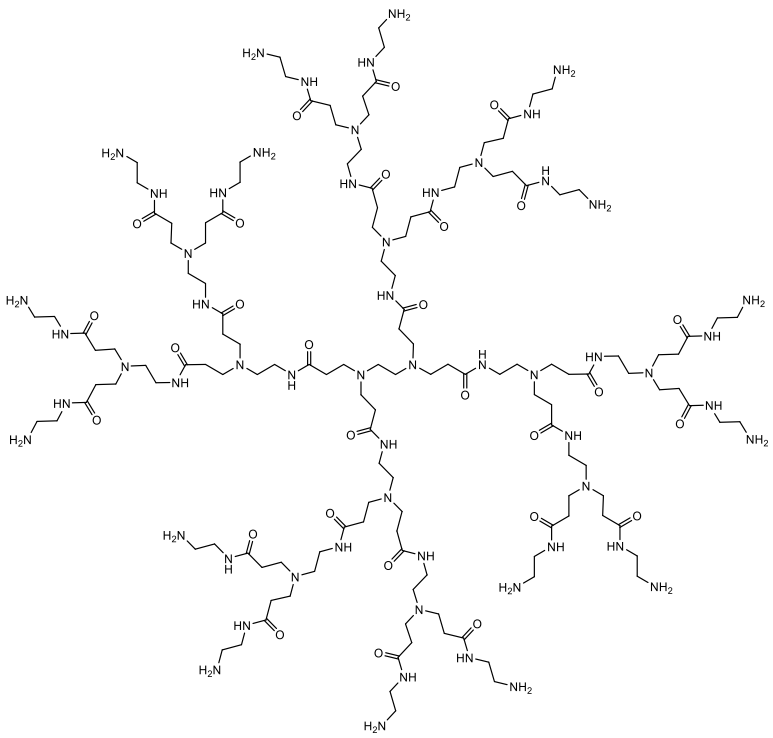


Figure 1.1 - Dendrimer synthetic strategy.

Dendrimers are composed of different structural features including the core, the branching unit and the surface group (Figure 1.2). The core of the dendrimer is usually isolated from the surrounding molecule. It has been used to protect sensitive groups, or insoluble groups such as catalyst[5] or even dye for imaging application.[6] The branching units are the monomers used in an iterative manner to grow the dendrimer. Some units are used more than others due to their ease of synthesis or their commercial availability. PAMAM dendrimers, shown in Scheme 1.1, were first developed by Tomalia[3] and are now commercially available.[7]



Scheme 1.1 – PAMAM dendrimer

Bis-MPA based dendrimers were developed and extensively studied by Hult and Malkoch[8] and are also commercially available. This class of monomers is of particular interest due to their intrinsic biocompatibility.[9] Finally, surface groups are important for the properties of the dendrimer, as they affect its solubility, and subsequent potential applications. Light harvesting groups can be used for photonic applications,[10] pharmaceutical agents for drug delivery,[11] dyes for imaging[12] or even combination of them to prepare multifunctional dendrimers.[13]

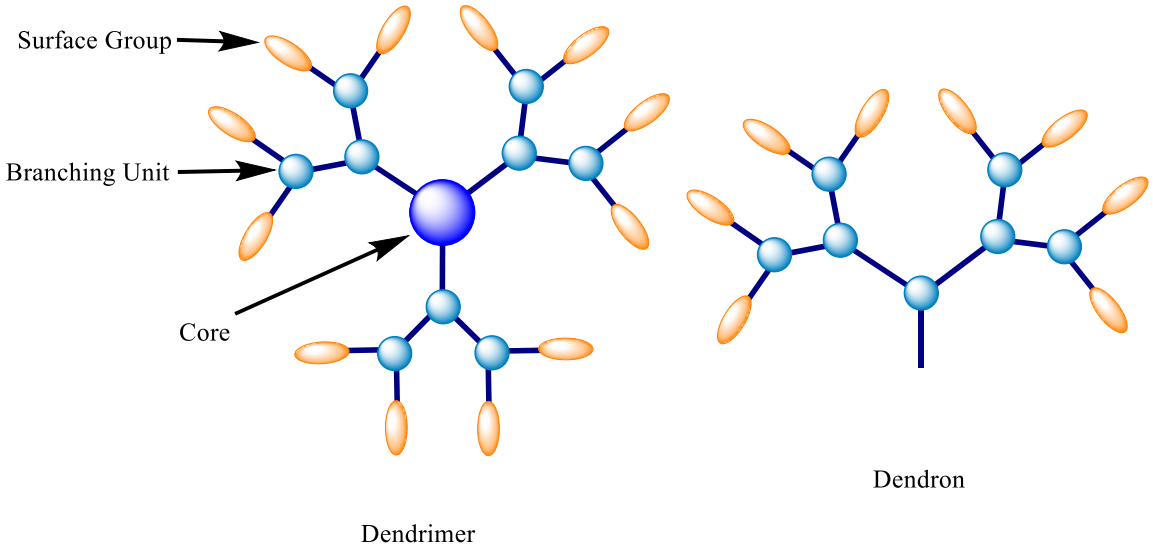


Figure 1.2 – Dendrimer and dendron structural feature.

As the interest in dendrimers was growing, other dendritic derivatives were developed, including dendrons (Figure 1.2). The later shares some of the properties of dendrimers, however they keep a reactive group at the core allowing covalent linkage with other types of molecules. Dendrons and dendrimers have been used extensively for functionalization of polymers, giving rise to new families of macromolecules (Figure 1.3) including dendronized polymers, star copolymers and linear dendritic block copolymers.[14]

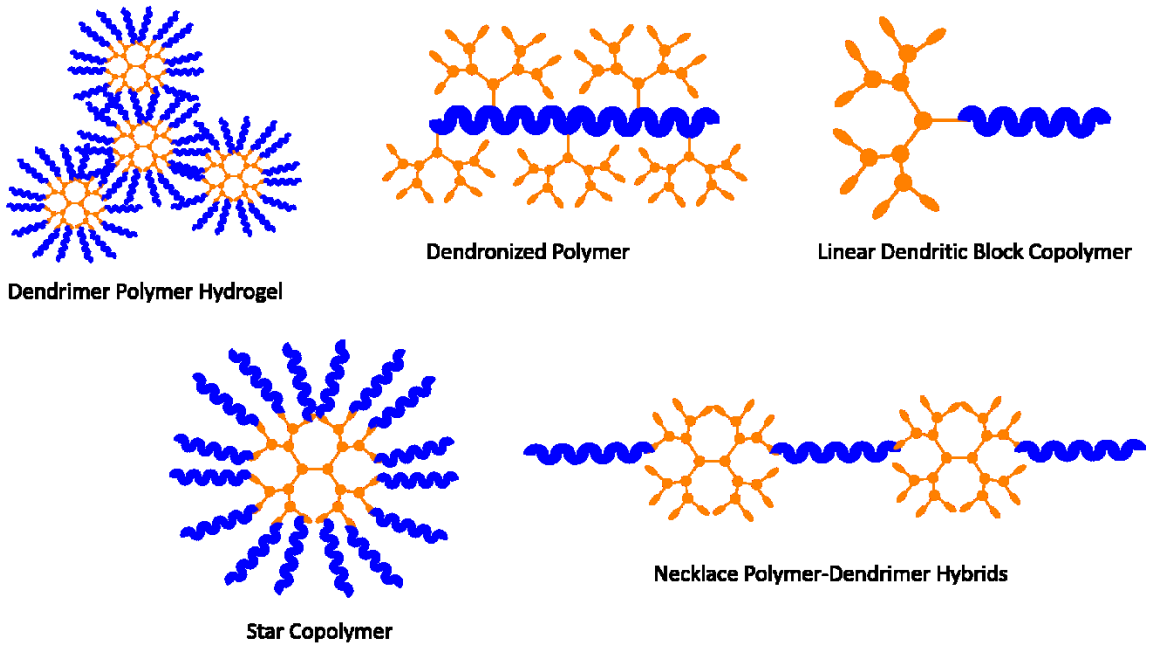


Figure 1.3 - Schematic representation of subclass of macromolecule combining the concept of dendrimer and polymer.

Star copolymers (Figure 1.3) are made of a dendrimer core on which the surface was functionalized with polymeric chains.[15] This class of macromolecules is extensively studied for their potential in drug delivery, and are sometimes referred to as unimolecular micelles, due to the dendritic core being hydrophobic and polymeric surface hydrophilic. Fréchet reported the functionalization of poly(benzylether) dendrimer with PEG, up to 48 surface groups for G4 dendrimer, and tested its drug loading capacity.[16] The results showed a modest loading, but did not prevent other groups to further explore the potential of this type of system varying the dendritic scaffold.[17]

Dendrimer polymer hydrogels are polymeric networks based on covalent cross-linking of dendrimer surface with polymers, they were first prepared by Gitsov and Zhu from "Fréchet-type" dendrimer and PEG.[18] The main advantage of this type of network

compared to polymeric hydrogels is the controlled number of cross-linking. In fact the higher the dendritic generation higher is the cross-linking.

Necklace polymer-dendrimer hybrid (Figure 1.3) is a theoretical class of macromolecules.[19] It has been proposed by Newkome,[20] and the closest example to this type of system was reported by Chow et al.. In their work, the dendrimer outer-shell is not connected through covalent linking with a polymer, but with a platinum complex, forming a supramolecular necklace polymer.[21]

Dendronized polymers are made of dendrons attached to a polymeric chain and thus share the properties of both.[22] They are not monodisperse, due to the polydispersity introduced by the polymer part. Furthermore, they are not globular in shape but instead are cylindrical in nature, with a diameter controlled by the dendron generation and a length control by the molecular weight of the polymer.[23] Polymers have the tendency to coil on themselves, but in a dendronized polymer due to the steric nature of the dendrons there is no possibility for this phenomenon. This effect gives the dendronized polymer a stiffness comparable to a rigid rod.[24] This class of macromolecules has a lot of potential applications, due to their large surface area, and have been used for example in catalysis[25] and biology.[26] Although not related to applications, it should be noted that the largest synthetic molecule ever prepared was a dendronized polymer, which is referred to as molecular object.[27] Molecular objects are mesoscopic molecules with a precise geometry and surface.[28] This molecule, prepared by Schluter et al., had a MW of 200MDa (200,000,000Da), possessed 17 million atoms, and had cylindrical shape with a diameter of 10 nm and length of a few microns (Figure 1.4).

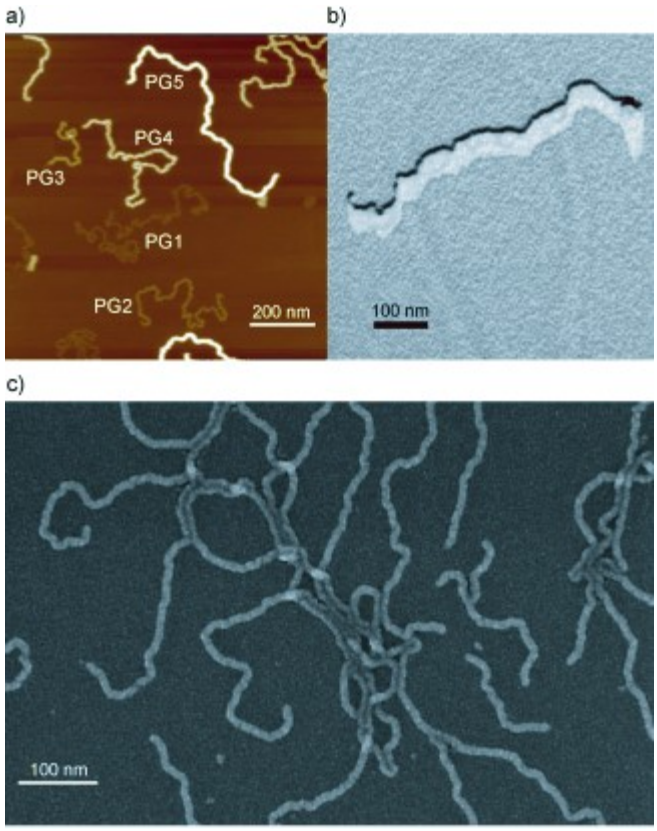


Figure 1.4 – AFM image (A) TEM image (B) and SEM image (C) of dendronized polymer prepared by Schluter et al.. Reprinted with permission from reference [27]. Copyright 2010 John Wiley and Sons.

1.2 – Linear Dendritic Block Copolymer

Linear dendritic block copolymers (LDBC), also referred to as dendrimer-polymer hybrids or telodendrimers, are a class of dendritic nanomaterials. They can be decomposed into two categories (Figure 1.5). The dumbbell dendrimer, which is ABA triblock macromolecule made of two dendrons (A) capping the two ends of the polymeric chain. On the other hand, the linear dendritic diblock copolymer is AB diblock macromolecule in which only one end of the polymer functionalized with the dendron.[19]

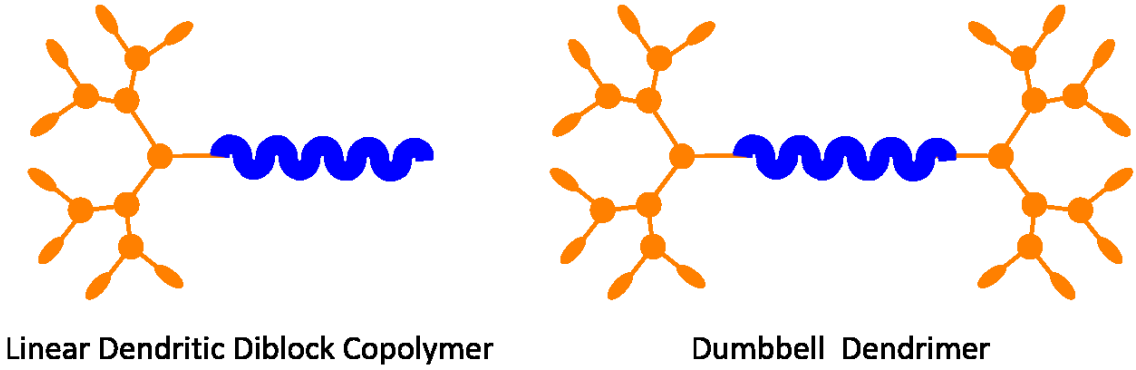
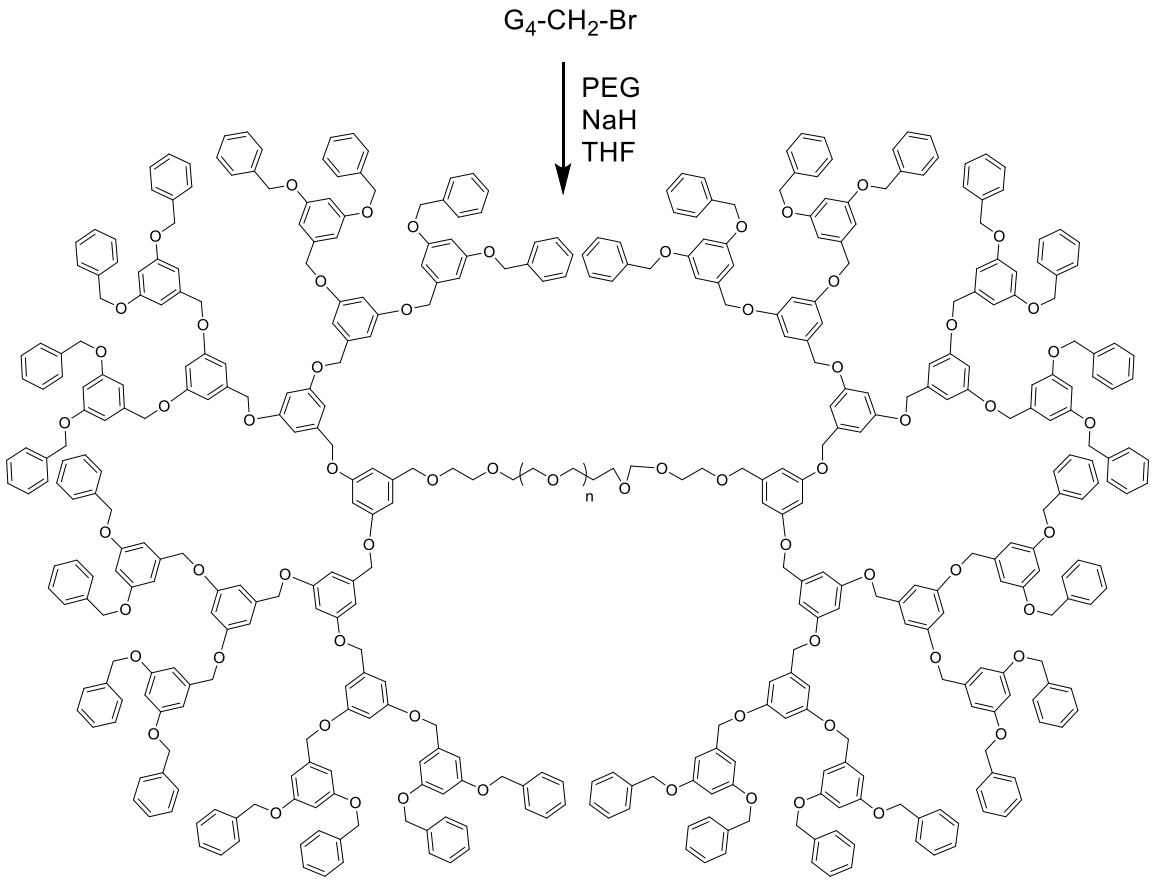


Figure 1.5 - Schematic representation of linear dendritic diblock copolymer and dumbbell dendrimer.

LDBC were introduced by Gitsov and Fréchet in 1992, with the synthesis of a LDBC made of poly(benzylether) dendron, also known as "Fréchet-type" dendron, and poly(ethylene glycol) (Scheme 1.2).[29] It was prepared by a coupling strategy, reacting the polymer with sodium hydride in presence of the dendron. Shortly after, Gitsov developed another synthetic methodology to prepare LDBC based on anionic polymerization of the polymer using the dendron as the macroinitiator.[30, 31] As the synthesis of LDBC was getting more accessible, new dendritic scaffolds were explored including poly(L-lysine),[32] PAMAM[33] and bis-MPA.[34] Other types of hybrid were also developed, Percec, among others,[35] replaced the linear polymer by a cyclic crown ether, giving rise to interesting self-assembly behavior.[36]



Scheme 1.2 - First LDBC synthesized by Gitsov and Fréchet.

LDBC can be synthesized using three different methods described in Figure 1.6.[37] The dendron first strategy in which the linear part is polymerized from the dendron which is used as an initiator.[38] The chain first strategy in which the dendron is grown from the polymeric chain.[39] Finally, the coupling method in which the dendron and the polymer are covalently linked to form the LDBC.[40] The first example of linear dendritic block copolymer[29] developed by Fréchet used this last approach. The limitations for the coupling method include a lower reactivity of the polymer and the dendron when MW of each part

is higher than 10,000 g/mol.[19] More recently, click chemistry has greatly expanded the scope of dendritic and polymeric scaffolds used.[41]

The chain first method, which is the most used one for LDBC synthesis, is restricted to low generation dendrimers. It is the most versatile method and has been employed in a variety of dendritic scaffolds including bis-MPA,[42] polyamidoamine (PAMAM),[33] carbosilane[43] and polyester.[44] The large molecular weight and the potential shielding effect of the polymer on the dendron reduce the reactivity in higher generation synthesis drastically.[45] Another important drawback of this method is the difficult characterization of the macromolecule. In the case of a large polymeric part, NMR spectroscopy and GPC are unlikely to show significant differences as the dendron generations increase, and MALDI-TOF is the only available tool to characterize these.[33, 45]

Finally, the dendron first method is the least used method. This method was developed by Fréchet in 1994[30] in which they used the dendron as the macroinitiator for polymerization. It has been mostly used in combination with "Fréchet-type" dendrons.[37] and has the advantage of not limiting the size of the dendron to lower generations.[30] This method has been used recently by Gitsov to prepare the first ABC dendritic-linear-dendritic triblock copolymer.[46]

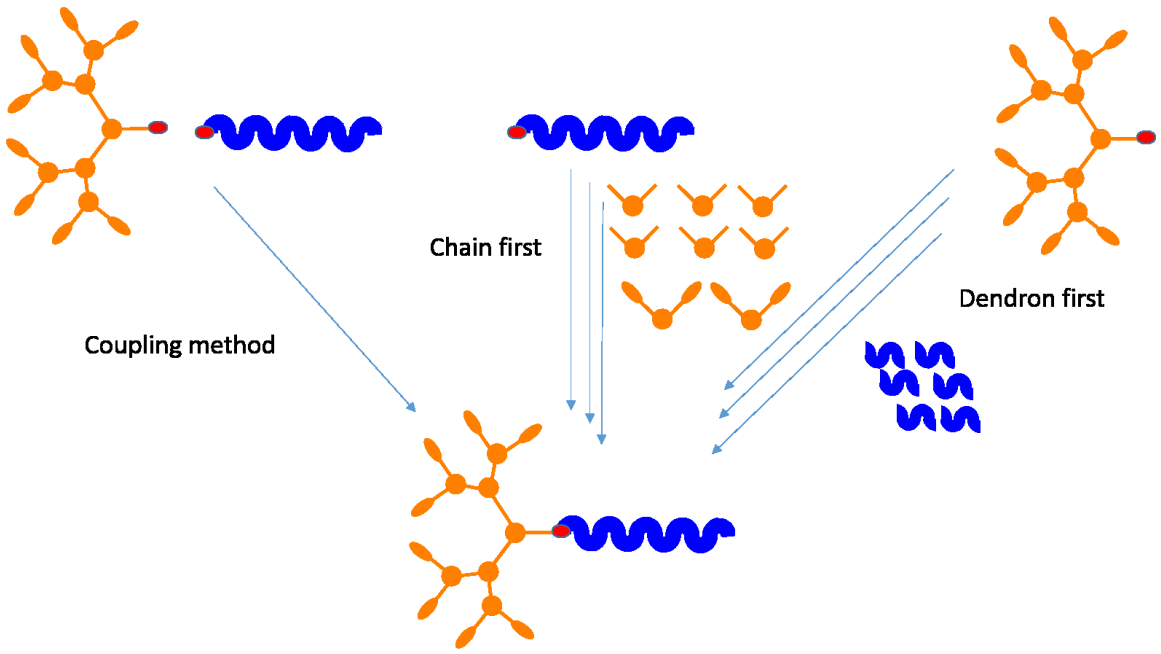


Figure 1.6 –Linear Dendritic Block Copolymer Synthetic Strategy.

1.3 - Applications

LDBCs have been used for a variety of applications including, catalysis[47] and drug delivery.[48] In 2002, Stupp and co-workers reported the synthesis of an ABC triblock dendritic-rod-coil copolymer.[49] The A block was based on aryl esters, 3,5 bis(hydroxyl)benzoic acid, the B block on rigid linear oligo(biphenyl ester) and the C block on the flexible oligoisoprene and oligobutadiene. The synthesis of this LDBC was carried out using both convergent and divergent synthesis of the dendron, and gave the desired macromolecules in high yield. Four years later, the same group reported the interesting properties of the generation 1 LDBC. In 15 different organic solvents and at a concentration as low as 0.2 weight% the LDBC acted as an organic gelator. In fact in DCM, higher concentration (>0.3%) resulted in loss of the gelation properties. The gel formation was due to the π-π interaction of the dendron backbone and the B block, as well as the H-

bonding of the dendron surface hydroxyl, resulting in the self-assembly of the LDBC in some organic solvents.[50]

LDBC can be appropriately functionalized and prepared to form hydrogels. Grinstaff and co-worker, prepared LDBC based hydrogels for biomedical application.[51] They tested different type of dendritic backbones based on succinic acid, β-alanine and glycerol, while keeping a PEG core as shown on Figure 1.7. By functionalizing the surface of the dendron with methylacrylic acid, they were able to irradiate with UV *in situ* to get the hydrogel fixed on the mouse tissue. Histological studies showed increase healing response on the treated injured mouse knee, compare to the non-treated knee.

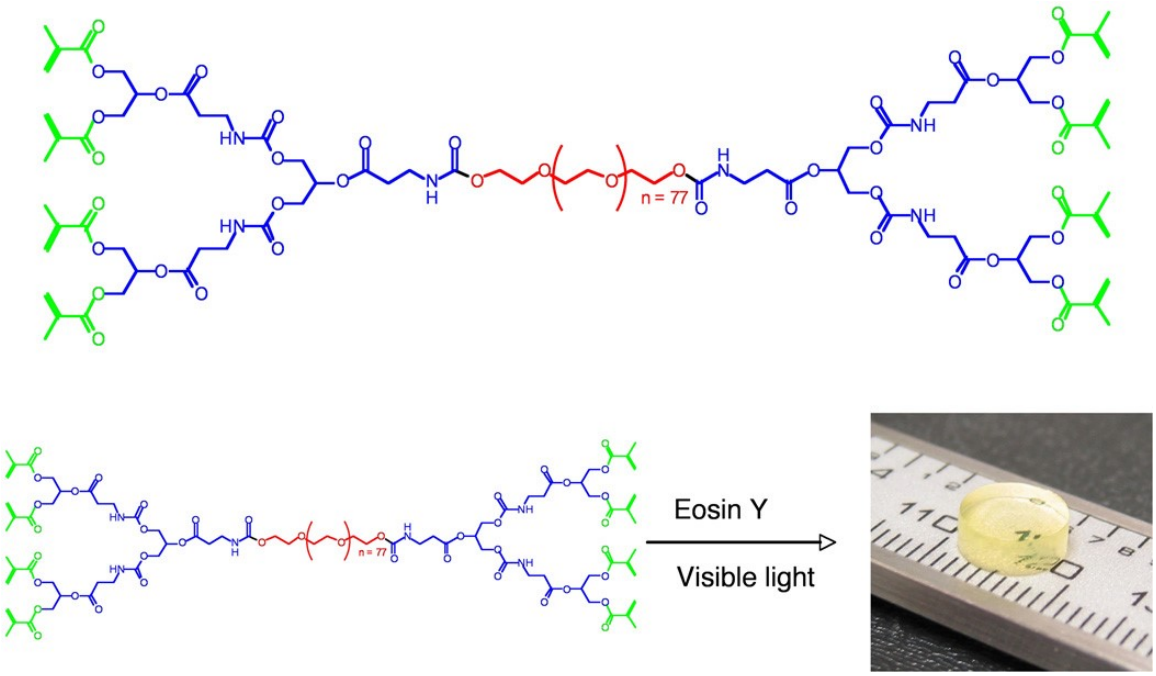


Figure 1.7 –Linear dendritic block copolymer for hydrogel preparation. Reprinted with permission from reference [51]. Copyright 2008 American Chemical Society.

Malkoch group has reported hydrogel preparation using dumbbell dendrimer bis-MPA dendrons a PEG chain. The bis-MPA dendrons were functionalized with allyl

moieties and subjected to UV irradiation in the presence of 3-mercaptopropionic acid to give novel type of dendritic hydrogels.[52] The bis-MPA/PEG scaffold was extensively studied by the group, for example they synthesized a linear dendritic diblock copolymer using this scaffold and functionalized the bis-MPA surface with poly(ε-caprolactone). This LDBC was then self-assembled using the co-solvent evaporation method to from micelle. They also tried drop casting technique of this LDBC on a glass plate to observe the microphase separation and obtained honeycomb membranes. These membranes were fully characterized and found to have a cavity of 3 μm large and 1 μm deep (Figure 1.8). This type of structure has a lot of potential for biological applications due to the large surface area.[34]

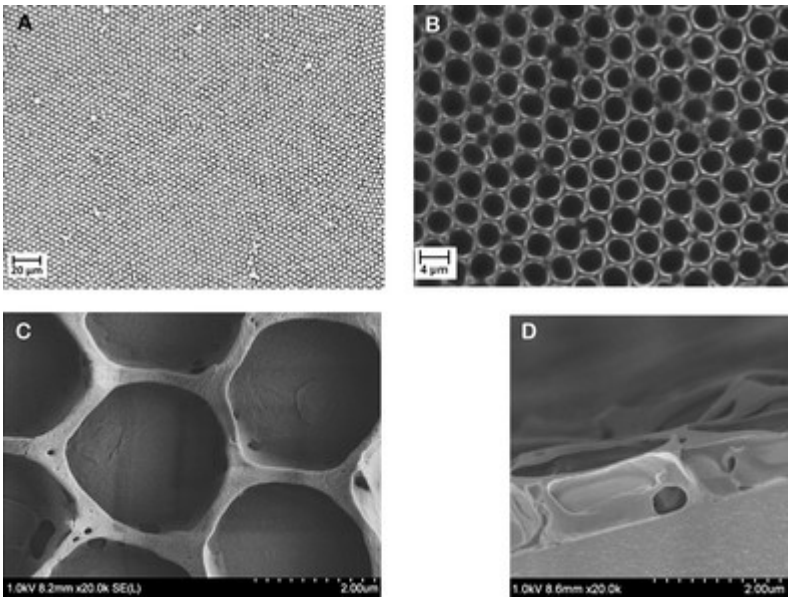


Figure 1.8 –Optical microscopy image (A and B) and SEM image (C and D) of Malkoch honeycomb membrane. Reprinted with permission of reference [46] Copyright 2011 Royal Society of Chemistry.

Amphiphilic LDBC, can easily self-assemble, either in bulk or in solution, and micelles are usually obtained, and have been extensively studied to encapsulate hydrophobic molecules.[34] The encapsulation mechanism in LDBC is significantly different than in conventional linear block copolymers.[19] Instead of staying at the interface of the core and the shell for linear block copolymer, hydrophobic molecules are able to get into the voids of the dendritic core of LDBC micelle, allowing large loading capacity.[53] This characteristic makes LDBC micelles suitable for drug delivery, since loading of large amounts of drug ensures therapeutic efficacy.

The high loading capacity has been exploited for optical applications. Wang et al. reported loading of lanthanide inside the core of a LDBC micelle prepared from poly(benzylether) dendron and poly(acrylic acid) chain. Lanthanides are known to have interesting optical properties and in this article the terbium 3+ ion (Tb^{3+}) was used. When loaded inside micelles, the fluorescence intensity of Tb^{3+} was increased. Furthermore, a strong dendritic effect was noticed with an increase of 5 fold of fluorescent intensity between generation 1 and 3 LDBC. The authors explained this optical behavior by the antenna effect of the dendron benzyl group and also by the microenvironment effect due to the lower number of water molecules coordinating the Tb^{3+} inside the micelle.[54]

Gitsov and co-workers used an LDBC to catalyse Diels-Alder reaction between fullerene C_{60} and anthracene. They were able to perform the reaction in green chemistry conditions, in water and at room temperature at an increased rate, due to LDBC micelle having high stability, low microviscosity and high loading capacity.[47]

LDBC can sometimes be functional without self-assembly, such as in the work reported by Chen and co-workers. Double hydrophilic LDBCs based on bis-MPA and PEG

were prepared, and the dendron functionalized with succinic anhydride to introduce a carboxylic acid on its surface, making it hydrophilic. They then tested the LDBC for biomineralization of $CaCO_3$, and showed that they were able to modify its crystallization, obtaining microspheres of $CaCO_3$.[55]

Finally, non-therapeutic biomedical applications have also been a subject of study for LDBC.[56] Brash and co-workers prepared a series of double hydrophilic dumbbell dendrimers with a PEG core and poly(L-lysine) dendron, as a CT scan contrast agent.[57] The problem with actual contrast agent is the short lifetime in the body, less than 5 minutes. By preparing large macromolecule, they extended the circulation time, and by functionalizing the dendron surface with triiodophtalamide, they were able to get good and prolonged signal on CT scan of an animal model.

1.4 - Gene delivery

Gene therapy holds tremendous potential for silencing gene overexpression in tumor cells, but the DNA used is readily degraded by the body, for this reason a delivery system is needed. PAMAM dendrimers have been extensively studied for this purpose and are known to be efficient vectors,[58] however due to their cytotoxicity, their potential is limited. A novel type of nanocarrier was proposed by Wagner and co-workers based on micellar diblock LDBC composed of PAMAM dendron and PEG. The surface of the dendron was functionalized with pentaethylenehexamine (PETA) to increase the interaction between the dendron and DNA and make the surface hydrophobic. The other end of PEG was functionalized with an epidermal growth factor (EGF) for active targeting of tumor cells, which generally overexpressed this receptor (Figure 1.9).[59] The self-

assembled micelle showed positive transfection results and higher transfection for EGF functionalized LDBC than non-functionalized LDBC.

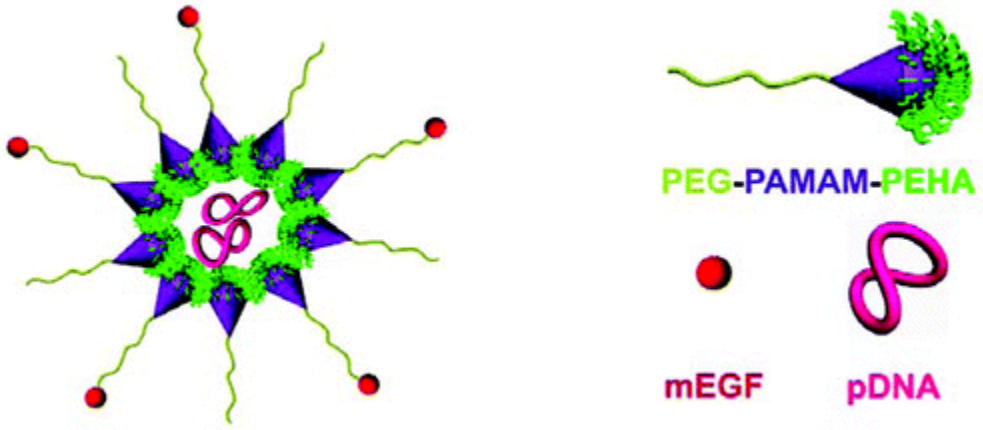


Figure 1.9 – Schematic representation of gene delivery system developed by Wagner and co-workers. Reprinted with permission from reference [59]. Copyright 2011 American Chemical Society.

Lin et al. pushed gene delivery further by codelivering doxorubicin, an anticancer drug, with the DNA. The LDBC is based on PAMAM to bind to the DNA and the linear part on poly-(N-ε-carbobenzyloxy-L-lysine) (PZLL). In this work, the dendron is hydrophilic and exposed on the surface of the micelle while PZLL is forming the hydrophobic core. The doxorubicin was loaded during micelle formation and thereafter the DNA was condensed on the micelle surface, the formation of the complex was verified by gel electrophoresis. The cytotoxicity of the system was tested and showed slight toxicity at high concentration, but lower than PAMAM dendrimers. The simultaneous delivery of DNA and the drug by fluoescense was examined and a good gene transfection of the system

was demonstrated.[60] Chen et al. reported a similar system to Lin but instead of delivering DNA, they used siRNA, which demonstrated equivalent efficiency.[61]

Other LDBC scaffolds have been tested for gene delivery, for example in an article from Cao et al., a dumbbell dendrimer composed of hydrophilic poly(L-lysine) dendron and hydrophobic poly(L-lactide) was prepared.[62] The self-assembled LDBC and DNA were complexed, to form the DNA nanocarrier, which was found to protect the DNA from DNase, and to have high transfection efficiency, and is thus a potential alternative to the more cytotoxic PAMAM.

1.5 - Drug delivery

For drug delivery applications, one of the most extensively studied LDBC scaffold is the bis-MPA/PEG. Gillies and Fréchet were the first to test in vitro the drug delivery by this type of LDBC. The surface of their bis-MPA dendron was functionalized with 3,5(dimethoxy)benzylidene, a pH sensitive group to control the delivery.[63] The results showed similar toxicty against cancer cells between the free drug, doxorubicin, and the loaded micelles. However, the doxorubicin distribution inside the cell was different, the drug accumulated in the nucleus when free, but when delivered through micelles it was located in intracellular organelles. This distribution difference might indicate a different mechanism of action.

Hu and co workers functionalized the dendron surface of bis-MPA/PEG with two different groups, either acetic acid or octadecanoic acid, and self-assembled the LDBC into micellar structures. They reported a lower CMC and smaller micelles for LDBC decorated

with the long aliphatic chains compared to acetic acid. Finally, they tested the biocompatibility of the system and showed no toxcity in cells.[64]

Recently, bis-MPA/PEG LDBC were also used by Jiang for combination therapy drug delivery.[65] The surface of the bis-MPA was decorated with trans-retinoic acid (Vitamin A) and the micelle was loaded with paclitaxel, an antitumoral agent generally used in breast cancer treatment (Figure 1.10). There were two reasons behind the choice of trans-retinoic acid, first it synergized therapeuticly with paclitaxel, second it allowed π-π stacking with itself to lower the CMC, and with the paclitaxel to increase drug loading. In fact, the CMC for this system is 3.48 mg/L which is low, thus the micelles were very stable. Biological studies were performed both *in vitro* and *in vivo,* and the results showed no synergy between the trans-retinoic acid and the paclitaxel. The drug delivery system also had poorer performance than paclitaxel alone both *in vitro* and *in vivo*. It was concluded that the π-π interactions were too strong and the paclitaxel was actually not released from the delivery system, which showed that π-π interaction might not be the ideal method to lower the CMC for drug delivery.

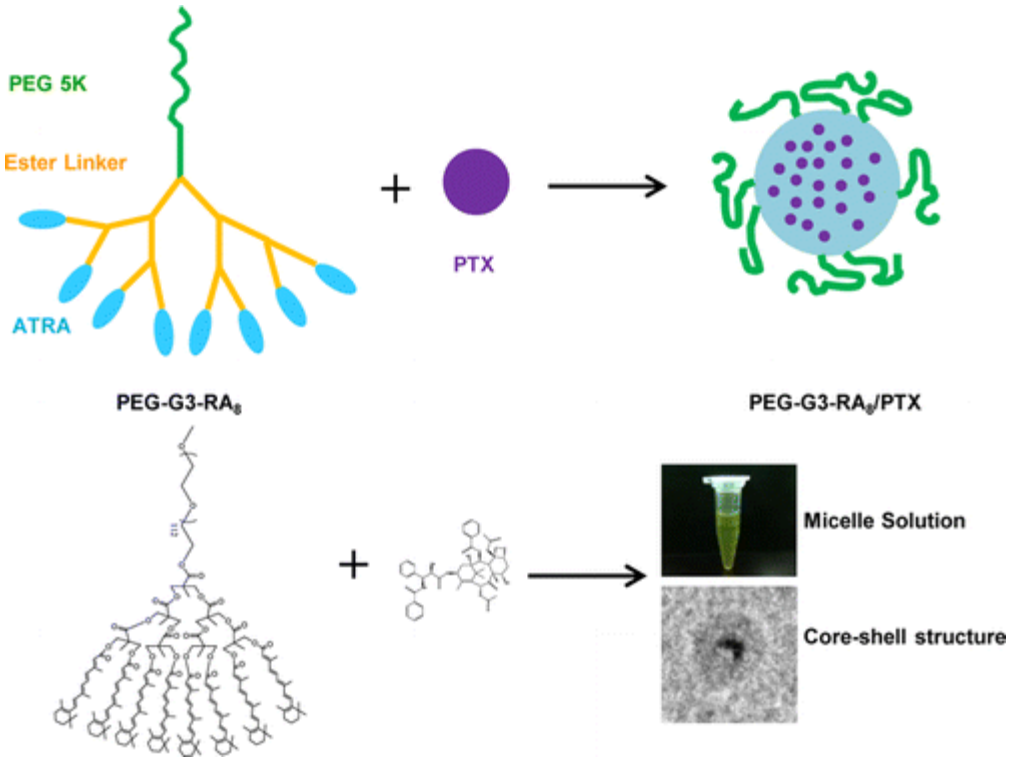


Figure 1.10 – Drug delivery system developed by Jiang and co-workers. Reprinted with permission from reference [65]. Copyright 2015 American Chemical Society.

In a very recent work from Luo et al., simulation was used to get the best possible dendritic surface for poly(L-lysine) dendron/PEG LDBC as a drug delivery system (Figure 1.11).[48] By calculating the interaction between a drug and several "drug binding moieties" Luo and co-workers were able to find the best surface group to cover the dendron, and thus increase drug loading inside micelle. After docking doxorubicin with 15 different drug binding moieties, they selected 8 different molecules to decorate LDBC and validate their computational predictions. The predictions were shown to be accurate in most cases and the rhein molecule, also known as cassic acid, functionalized micellar LDBC showed the best loading capacity. They performed *in vivo* and *in vitro* studies of the loaded micelle,

showing decrease in side effects, increased tumor-targeting and increased anticancer activity compared to free doxorubicin. This study is the first example of *de novo* design of LDBC for drug delivery applications.

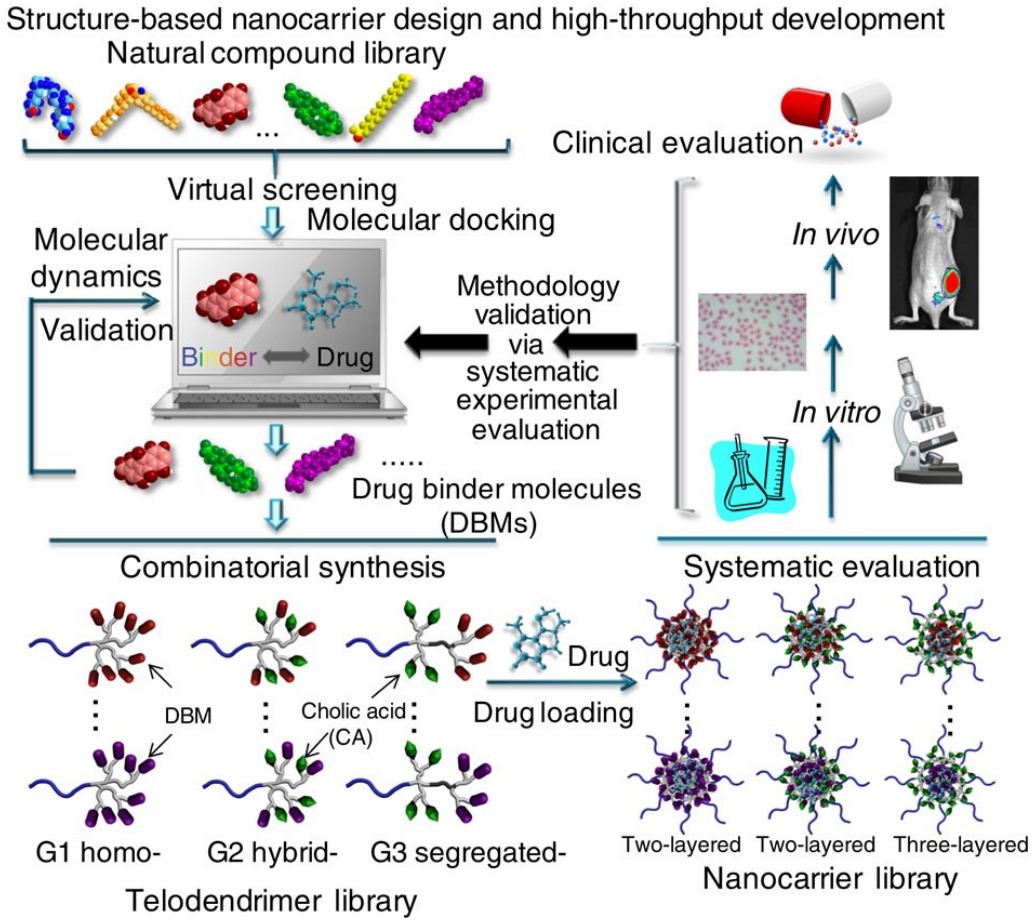


Figure 1.11 – Schematic representation of the methodology developed by Luo et al. for nanocarrier design. Reprinted with permission from reference [48].

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1.6 - Goals

The goals of this thesis are to develop a simple and versatile methodology to synthesize a series of pH responsive amphiphilic linear dendritic block copolymers, examine their self-assembly and evaluate their efficiency as drug delivery systems. The synthesis is based on the use of Steglish esterification and click chemistry to prepare LDBC through coupling of the poly(ethylene glycol) polymer to the bis-MPA dendron, while the pH responsiveness of the system will be brought in by the surface functionalization of the dendron with acetonide group. We shall employ high yield reactions including click chemistry for the construction of dendrons on a prentaerythritol core which will be subsequently coupled to a linear poly(ethylene glycol) chain. The pH sensitivity of the LDBC will be ensured by a simple and easily prepared acetonide group. The self-assembly will be carried out by co-solvent evaporation method and the resulting nanostructures will be analysed by DLS and TEM techniques. The pH responsiveness of the self-assembled LDBC will also be studied using these two techniques. pH response studies of micelle are usually only carried out by DLS, however by complementing the DLS study with TEM imaging, we will be able to obtain a better understanding of this phenomenom. The ultimate goal of this project is to develop a highly efficient drug delivery system, and in this vein, we shall examine loading efficiency of self-assembled LDBC by encapsulating acetalozamide and testing its efficacy *in vitro* against cancer cell spheroids. Acetalozamide is a drug with significant potential in cancer treatment, however it has never been used inside a drug delivery system for this purpose. This will be the first example of *in vitro* study of acetalozamide loaded LDBC micelles.

1.7 - References

1. Buhleier, E.; Wehner, W.; Vögtle, F., "Cascade"-and" Nonskid-Chain-like" Syntheses of Molecular Cavity Topologies. *Synthesis* **1978**, (2), 155-158
2. Hourani, R.; Kakkar, A., Advances in the Elegance of Chemistry in Designing Dendrimers. *Macromol. Rapid Commun.* **2010,** *31* (11), 947-974.
3. Tomalia, D. A.; Baker, H.; Dewald, J.; Hall, M.; Kallos, G.; Martin, S.; Roeck, J.; Ryder, J.; Smith, P., A New Class of Polymers: Starburst-Dendritic Macromolecules. *Polym J* **1985,** *17* (1), 117-132.
4. Hawker, C. J.; Frechet, J. M. J., Preparation of polymers with controlled molecular architecture. A new convergent approach to dendritic macromolecules. *J. Am. Chem. Soc.* **1990,** *112* (21), 7638-7647.
5. Deraedt, C.; Pinaud, N.; Astruc, D., Recyclable Catalytic Dendrimer Nanoreactor for Part-Per-Million CuI Catalysis of "Click" Chemistry in Water. *J. Am. Chem. Soc.* **2014,** *136* (34), 12092-12098.
6. Mao, M.; Song, Q.-H., Non-conjugated dendrimers with a porphyrin core and coumarin chromophores as peripheral units: Synthesis and photophysical properties. *Dyes and Pigments* **2012,** *92* (3), 975-981.
7. Esfand, R.; Tomalia, D. A., Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. *Drug Discovery Today* **2001,** *6* (8), 427-436.
8. Carlmark, A.; Malmstrom, E.; Malkoch, M., Dendritic architectures based on bis-MPA: functional polymeric scaffolds for application-driven research. *Chem. Soc. Rev.* **2013,** *42* (13), 5858-5879.
9. Feliu, N.; Walter, M. V.; Montañez, M. I.; Kunzmann, A.; Hult, A.; Nyström, A.; Malkoch, M.; Fadeel, B., Stability and biocompatibility of a library of polyester dendrimers in comparison to polyamidoamine dendrimers. *Biomaterials* **2012,** *33* (7), 1970-1981.
10. Adronov, A.; Frechet, J. M. J., Light-harvesting dendrimers. *Chem. Commun.* **2000,** (18), 1701-1710.
11. Sharma, A.; Gautam, S. P.; Gupta, A. K., Surface modified dendrimers: Synthesis and characterization for cancer targeted drug delivery. *Biorg. Med. Chem.* **2011,** *19* (11), 3341-3346.
12. Kim, Y.; Kim, Sung H.; Tanyeri, M.; Katzenellenbogen, John A.; Schroeder, Charles M., Dendrimer Probes for Enhanced Photostability and Localization in Fluorescence Imaging. *Biophys. J.* **2013,** *104* (7), 1566-1575.
13. Sharma, A.; Khatchadourian, A.; Khanna, K.; Sharma, R.; Kakkar, A.; Maysinger, D., Multivalent niacin nanoconjugates for delivery to cytoplasmic lipid droplets. *Biomaterials* **2011,** *32* (5), 1419-1429; Sharma, A.; Soliman, G. M.; Al-Hajaj, N.; Sharma, R.; Maysinger, D.; Kakkar, A., Design and Evaluation of Multifunctional Nanocarriers for Selective Delivery of Coenzyme Q10 to Mitochondria. *Biomacromolecules* **2012,** *13* (1), 239-252.
14. Frauenrath, H., Dendronized polymers—building a new bridge from molecules to nanoscopic objects. *Prog. Polym. Sci.* **2005,** *30* (3–4), 325-384.
15. Lapienis, G., Star-shaped polymers having PEO arms. *Prog. Polym. Sci.* **2009,** *34* (9), 852-892.

16. Liu, M.; Kono, K.; Fréchet, J. M. J., Water-soluble dendritic unimolecular micelles:: Their potential as drug delivery agents. *J. Controlled Release* **2000,** *65* (1–2), 121-131.
17. Atanasov, V.; Sinigersky, V.; Klapper, M.; Müllen, K., Core−Shell Macromolecules with Rigid Dendritic Polyphenylene Cores and Polymer Shells. *Macromolecules* **2005,** *38* (5), 1672-1683; Kojima, C.; Kono, K.; Maruyama, K.; Takagishi, T., Synthesis of Polyamidoamine Dendrimers Having Poly(ethylene glycol) Grafts and Their Ability To Encapsulate Anticancer Drugs. *Bioconjugate Chem.* **2000,** *11* (6), 910-917.
18. Gitsov, I.; Zhu, C., Amphiphilic Hydrogels Constructed by Poly(ethylene glycol) and Shape-Persistent Dendritic Fragments1. *Macromolecules* **2002,** *35* (22), 8418-8427.
19. Gitsov, I., Hybrid linear dendritic macromolecules: From synthesis to applications. *J. Polym. Sci. A Polym. Chem.* **2008,** *46* (16), 5295-5314.
20. Newkome, G. R.; Moorefield, C. N.; Vögtle, F., Dendritic Networks. In *Dendrimers and Dendrons*, Wiley-VCH Verlag GmbH & Co. KGaA: 2004; pp 539-562.
21. Chow, H.-F.; Leung, C.-F.; Li, W.; Wong, K.-W.; Xi, L., Synthesis and Characterization of Dendritic Necklaces: A Class of Outer-Sphere–Outer-Sphere Connected Dendronized Organoplatinum Polymers. *Angew. Chem. Int. Ed.* **2003,** *42* (40), 4919-4923.
22. Khan, A.; Zhang, B.; Schlüter, A. D., Dendronized Polymers: An Approach to Single Molecular Objects. In *Mater. Sci. Technol.*, Wiley-VCH Verlag GmbH & Co. KGaA: 2006.
23. Campagna, S.; Ceroni, P.; Puntoriero, F. Designing dendrimers.
24. Zhang, A.; Shu, L.; Bo, Z.; Schlüter, A. D., Dendronized Polymers: Recent Progress in Synthesis. *Macromol. Chem. Phys.* **2003,** *204* (2), 328-339.
25. Liang, C. O.; Helms, B.; Hawker, C. J.; Frechet, J. M. J., Dendronized cyclocopolymers with a radial gradient of polarity and their use to catalyze a difficult esterification. *Chem. Commun.* **2003,** (20), 2524-2525.
26. Ronda, J. C.; Reina, J. A.; Giamberini, M., Self-organized liquid-crystalline polyethers obtained by grafting tapered mesogenic groups onto poly(epichlorohydrin): Toward biomimetic ion channels 2. *J. Polym. Sci. A Polym. Chem.* **2004,** *42* (2), 326-340; Hollins, A.; Benboubetra, M.; Omidi, Y.; Zinselmeyer, B.; Schatzlein, A.; Uchegbu, I.; Akhtar, S., Evaluation of Generation 2 and 3 Poly(Propylenimine) Dendrimers for the Potential Cellular Delivery of Antisense Oligonucleotides Targeting the Epidermal Growth Factor Receptor. *Pharm. Res.* **2004,** *21* (3), 458-466.
27. Zhang, B.; Wepf, R.; Fischer, K.; Schmidt, M.; Besse, S.; Lindner, P.; King, B. T.; Sigel, R.; Schurtenberger, P.; Talmon, Y.; Ding, Y.; Kröger, M.; Halperin, A.; Schlüter, A. D., The Largest Synthetic Structure with Molecular Precision: Towards a Molecular Object. *Angew. Chem. Int. Ed.* **2011,** *50* (3), 737-740.
28. Schlüter, A. D.; Halperin, A.; Kröger, M.; Vlassopoulos, D.; Wegner, G.; Zhang, B., Dendronized Polymers: Molecular Objects between Conventional Linear Polymers and Colloidal Particles. *ACS Macro Letters* **2014,** *3* (10), 991-998.
29. Gitsov, I.; Wooley, K. L.; Fréchet, J. M. J., Novel Polyether Copolymers Consisting of Linear and Dendritic Blocks. *Angew. Chem. Int. Ed. in English* **1992,** *31* (9), 1200-1202.

30. Gitsov, I.; Ivanova, P. T.; Fréchet, J. M. J., Dendrimers as macroinitiators for anionic ring-opening polymerization. Polymerization of ε-caprolactone. *Macromol. Rapid Commun.* **1994,** *15* (5), 387-393.
31. Gitsov, I.; Frechet, J. M. J., Novel Nanoscopic Architectures. Linear-Globular ABA Copolymers with Polyether Dendrimers as A Blocks and Polystyrene as B Block. *Macromolecules* **1994,** *27* (25), 7309-7315.
32. Chapman, T. M.; Hillyer, G. L.; Mahan, E. J.; Shaffer, K. A., Hydraamphiphiles: Novel Linear Dendritic Block Copolymer Surfactants. *J. Am. Chem. Soc.* **1994,** *116* (24), 11195-11196.
33. Iyer, J.; Fleming, K.; Hammond, P. T., Synthesis and Solution Properties of New Linear-Dendritic Diblock Copolymers. *Macromolecules* **1998,** *31* (25), 8757-8765.
34. Lundberg, P.; Walter, M. V.; Montanez, M. I.; Hult, D.; Hult, A.; Nystrom, A.; Malkoch, M., Linear dendritic polymeric amphiphiles with intrinsic biocompatibility: synthesis and characterization to fabrication of micelles and honeycomb membranes. *Polym. Chem.* **2011,** *2* (2), 394-402.
35. Gitsov, I.; Ivanova, P. T., Synthesis of new hybrid macromolecules with cyclo-dendritic architecture. *Chem. Commun.* **2000,** (4), 269-270.
36. Percec, V.; Johansson, G.; Ungar, G.; Zhou, J., Fluorophobic Effect Induces the Self-Assembly of Semifluorinated Tapered Monodendrons Containing Crown Ethers into Supramolecular Columnar Dendrimers Which Exhibit a Homeotropic Hexagonal Columnar Liquid Crystalline Phase. *J. Am. Chem. Soc.* **1996,** *118* (41), 9855-9866.
37. Wurm, F.; Frey, H., Linear–dendritic block copolymers: The state of the art and exciting perspectives. *Prog. Polym. Sci.* **2011,** *36* (1), 1-52.
38. Matyjaszewski, K.; Shigemoto, T.; Fréchet, J. M. J.; Leduc, M., Controlled/"Living" Radical Polymerization with Dendrimers Containing Stable Radicals. *Macromolecules* **1996,** *29* (12), 4167-4171.
39. Yim, S.-H.; Huh, J.; Ahn, C.-H.; Park, T. G., Development of a Novel Synthetic Method for Aliphatic Ester Dendrimers. *Macromolecules* **2007,** *40* (2), 205-210.
40. Chen, T.; Wang, L.; Jiang, G.; Wang, J.; Wang, X. J.; Zhou, J.; Wang, J.; Chen, C.; Wang, W.; Gao, H., Electrochemical behavior on poly(ferrocenyldimethylsilane)-b-poly(benzyl ether) linear-dendritic organometallic polymer films. *J. Electroanal. Chem.* **2006,** *586* (1), 122-127.
41. Hua, C.; Peng, S.-M.; Dong, C.-M., Synthesis and Characterization of Linear-Dendron-like Poly(ε-caprolactone)-b-poly(ethylene oxide) Copolymers via the Combination of Ring-Opening Polymerization and Click Chemistry. *Macromolecules* **2008,** *41* (18), 6686-6695.
42. Ihre, H.; Padilla De Jesús, O. L.; Fréchet, J. M. J., Fast and Convenient Divergent Synthesis of Aliphatic Ester Dendrimers by Anhydride Coupling. *J. Am. Chem. Soc.* **2001,** *123* (25), 5908-5917.
43. Chang, Y.; Kim, C., Synthesis and photophysical characterization of amphiphilic dendritic–linear–dendritic block copolymers. *J. Polym. Sci. A Polym. Chem.* **2001,** *39* (6), 918-926.
44. Carnahan, M. A.; Middleton, C.; Kim, J.; Kim, T.; Grinstaff, M. W., Hybrid Dendritic−Linear Polyester−Ethers for in Situ Photopolymerization. *J. Am. Chem. Soc.* **2002,** *124* (19), 5291-5293.

45. Choi, J. S.; Joo, D. K.; Kim, C. H.; Kim, K.; Park, J. S., Synthesis of a Barbell-like Triblock Copolymer, Poly(l-lysine) Dendrimer-block-Poly(ethylene glycol)-block-Poly(l-lysine) Dendrimer, and Its Self-Assembly with Plasmid DNA. *J. Am. Chem. Soc.* **2000,** *122* (3), 474-480.
46. Gitsov, I.; Simonyan, A.; Vladimirov, N. G., Synthesis of novel asymmetric dendritic-linear-dendritic block copolymers via "living" anionic polymerization of ethylene oxide initiated by dendritic macroinitiators. *J. Polym. Sci. A Polym. Chem.* **2007,** *45* (22), 5136-5148.
47. Simonyan, A.; Gitsov, I., Linear-Dendritic Supramolecular Complexes as Nanoscale Reaction Vessels for "Green" Chemistry. Diels−Alder Reactions between Fullerene C60 and Polycyclic Aromatic Hydrocarbons in Aqueous Medium. *Langmuir* **2008,** *24* (20), 11431-11441.
48. Shi, C.; Guo, D.; Xiao, K.; Wang, X.; Wang, L.; Luo, J., A drug-specific nanocarrier design for efficient anticancer therapy. *Nat Commun* **2015,** *6*.
49. Zubarev, E. R.; Stupp, S. I., Dendron Rodcoils: Synthesis of Novel Organic Hybrid Structures. *J. Am. Chem. Soc.* **2002,** *124* (20), 5762-5773.
50. Zubarev, E. R.; Sone, E. D.; Stupp, S. I., The Molecular Basis of Self-Assembly of Dendron–Rod–Coils into One-Dimensional Nanostructures. *Chemistry – A European Journal* **2006,** *12* (28), 7313-7327.
51. Degoricija, L.; Bansal, P. N.; Söntjens, S. H. M.; Joshi, N. S.; Takahashi, M.; Snyder, B.; Grinstaff, M. W., Hydrogels for Osteochondral Repair Based on Photocrosslinkable Carbamate Dendrimers. *Biomacromolecules* **2008,** *9* (10), 2863-2872.
52. Andrén, O. C. J.; Walter, M. V.; Yang, T.; Hult, A.; Malkoch, M., Multifunctional Poly(ethylene glycol): Synthesis, Characterization, and Potential Applications of Dendritic–Linear–Dendritic Block Copolymer Hybrids. *Macromolecules* **2013,** *46* (10), 3726-3736.
53. Gitsov, I.; Lambrych, K. R.; Remnant, V. A.; Pracitto, R., Micelles with highly branched nanoporous interior: Solution properties and binding capabilities of amphiphilic copolymers with linear dendritic architecture. *J. Polym. Sci. A Polym. Chem.* **2000,** *38* (15), 2711-2727.
54. Zhu, L.; Tong, X.; Li, M.; Wang, E., Luminescence Enhancement of Tb3+ Ion in Assemblies of Amphiphilic Linear−Dendritic Block Copolymers: Antenna and Microenvironment Effects. *J. Phys. Chem. B* **2001,** *105* (12), 2461-2464.
55. Wang, L.; Meng, Z.; Yu, Y.; Meng, Q.; Chen, D., Synthesis of hybrid linear-dendritic block copolymers with carboxylic functional groups for the biomimetic mineralization of calcium carbonate. *Polymer* **2008,** *49* (5), 1199-1210.
56. Chen, W.-T.; Thirumalai, D.; Shih, T.-F.; Chen, R.-C.; Tu, S.-Y.; Lin, C.-I.; Yang, P.-C., Dynamic Contrast-Enhanced Folate-Receptor-Targeted MR Imaging Using a Gd-loaded PEG-Dendrimer–Folate Conjugate in a Mouse Xenograft Tumor Model. *Mol Imaging Biol* **2010,** *12* (2), 145-154.
57. Fu, Y.; Nitecki, D. E.; Maltby, D.; Simon, G. H.; Berejnoi, K.; Raatschen, H.-J.; Yeh, B. M.; Shames, D. M.; Brasch, R. C., Dendritic Iodinated Contrast Agents with PEG-Cores for CT Imaging: Synthesis and Preliminary Characterization. *Bioconjugate Chem.* **2006,** *17* (4), 1043-1056.
58. Amiji, *Cationic Dendrimers as Gene Transfection Vectors*. CRC Press: 2004.

59. Yu, H.; Nie, Y.; Dohmen, C.; Li, Y.; Wagner, E., Epidermal Growth Factor–PEG Functionalized PAMAM-Pentaethylenehexamine Dendron for Targeted Gene Delivery Produced by Click Chemistry. *Biomacromolecules* **2011,** *12* (6), 2039-2047.
60. Lin, J.-T.; Zou, Y.; Wang, C.; Zhong, Y.-C.; Zhao, Y.; Zhu, H.-E.; Wang, G.-H.; Zhang, L.-M.; Zheng, X.-B., Cationic micellar nanoparticles for DNA and doxorubicin co-delivery. *Materials Science and Engineering: C* **2014,** *44*, 430-439.
61. Zhang, Y.; Chen, J.; Xiao, C.; Li, M.; Tian, H.; Chen, X., Cationic Dendron-Bearing Lipids: Investigating Structure–Activity Relationships for Small Interfering RNA Delivery. *Biomacromolecules* **2013,** *14* (12), 4289-4300.
62. Li, Y.; Cui, L.; Li, Q.; Jia, L.; Xu, Y.; Fang, Q.; Cao, A., Novel Symmetric Amphiphilic Dendritic Poly(l-lysine)-b-Poly(l-lactide)-b-Dendritic Poly(l-lysine) with High Plasmid DNA Binding Affinity as a Biodegradable Gene Carrier. *Biomacromolecules* **2007,** *8* (5), 1409-1416.
63. Gillies, E. R.; Fréchet, J. M. J., pH-Responsive Copolymer Assemblies for Controlled Release of Doxorubicin. *Bioconjugate Chem.* **2005,** *16* (2), 361-368.
64. Zhang, W.; Jiang, W.; Zhang, D.; Bai, G.; Lou, P.; Hu, Z., Synthesis, characterization and association behavior of linear-dendritic amphiphilic diblock copolymers based on poly(ethylene oxide) and a dendron derived from 2,2-bis(hydroxymethyl)propionic acid. *Polym. Chem.* **2015,** *6* (12), 2274-2282.
65. Li, J.; Jiang, X.; Guo, Y.; An, S.; Kuang, Y.; Ma, H.; He, X.; Jiang, C., Linear-dendritic copolymer composed of polyethylene glycol and all-trans-retinoic acid as drug delivery platform for paclitaxel against breast cancer. *Bioconjugate Chem.* **2015,** *26* (3), 418-26.

Chapter 2: Design and Synthesis of Linear Dendritic Block Copolymers

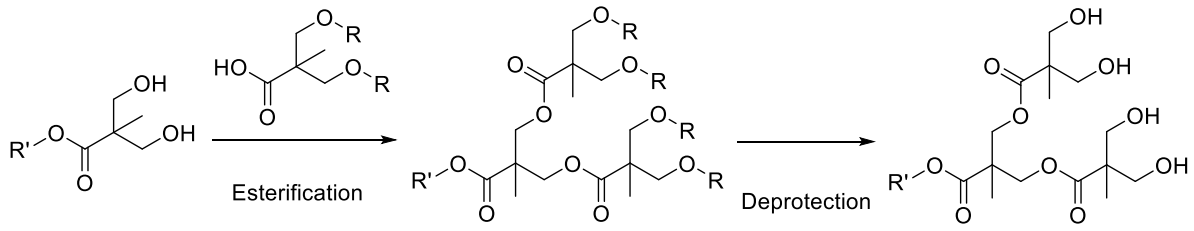
2.1 - Introduction

Linear dendritic block copolymers (LDBC) constitute a new addition to the family of macromolecules, and are composed of two blocks, a dendron and a linear polymer.[1] The diblock structure allows the preparation of amphiphilic macromolecules, which can then be self-assembled into various nanostructures.[2] In our study, we want to prepare pH responsive amphiphilic linear dendritic block copolymers for drug delivery. The amphiphilic character of the LDBC is achieved with a hydrophobic dendron based on 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) and a hydrophilic polymeric chain, poly(ethylene glycol) (PEG), which are both known for their biocompatibility.[3, 4] The dendron offers a unique platform to introduce functionalities on the macromolecule, that can affect the self-assembled nanostructures.[5] We used this opportunity to decorate the dendron with acetal groups to prepared pH-responsive nanostructure.

Poly(ethylene glycol) is a biocompatible water soluble polymer made of multiple units of ethylene glycol.[3] It has a wide variety of applications in medicinal chemistry[6] including for example, as a drug solubilizing agent[7] or a protein conjugate[8]. PEG is used in this project as it brings stealth, improved solubility, and colloidal stability to the drug delivery system[9]. It has been extensively employed in designing amphiphilic systems for drug delivery[10], and even in the conception of linear dendritic diblock copolymer.[11]

The bis-MPA molecule **(1)**, used in constructing the dendron, is an AB_2 type of monomer composed of functional groups, a carboxylic acid and two hydroxyls at opposite

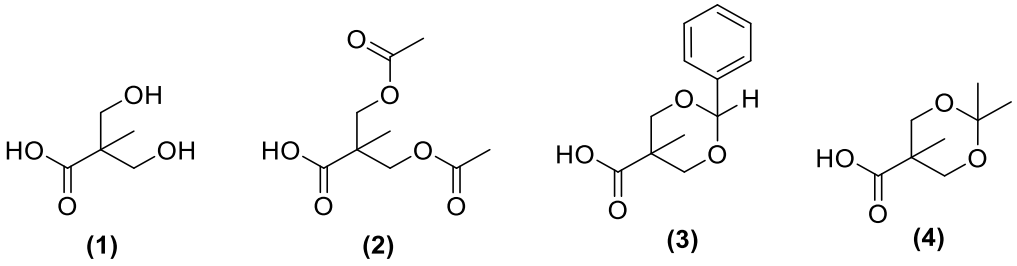
end (Scheme 2.2). It is a widely used scaffold[12] based on the esterification of the carboxylic acid with a hydroxyl group and the protection and deprotection of the diol (Scheme 2.1). The bis-MPA molecule is hydrophobic when its diols are protected. In addition to its well-established chemistry, bis-MPA is also biocompatible. In fact, bis-MPA dendritic molecules have been demonstrated to be biocompatible, contrary to other popular dendritic scaffolds,[13] which make them valuable for biomedical applications.[4]



Scheme 2.1 – Bis-MPA dendritic growth.

Bis-MPA chemistry was first introduced by Hult et al. in 1993,[14] who used this building block for the synthesis of hyperbranched polymers. In 1996, the same group prepared the first bis-MPA dendrimer[15]. However, there were two major problems with this dendritic scaffold. First, the acetate protecting groups **(2)** were not cleavable, making the surface functionalization of the dendrimer difficult (Scheme 2.2). Second, the synthesis was done through convergent assembly of the dendrimer, limiting the dendrimer to lower generations. In 1998, Hult et al. developed a new chemistry based on acetonide protection of the bis-MPA's diols **(4)** (Scheme 2.2). The esterification was carried out using dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridinium-4-toluene-sulfonate (DTPS) and the deprotection using a cationic resin (Dowex).[16] This method had the double advantage of i) allowing easy functionalization of the surface and ii) unabling the divergent synthesis of bis-MPA dendrimer. However, DCC was not the ideal reagent to use, since it is toxic and hard to separate from the product.[17] Fréchet subsequently proposed the use of

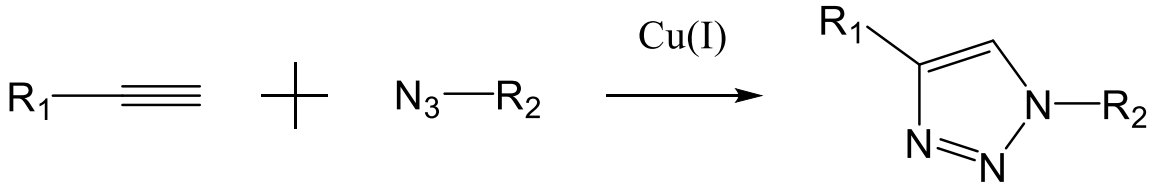
anhydride bis-MPA to increase the generation number.[18] They also proposed changing the protecting group to a benzylidene **(3)** which is more stable than the acetonide group, but needs hydrogenation to be deprotected (Scheme 2.2). In 2002, Malkoch took advantage of both the efficient anhydride coupling, and easy deprotection of the acetonide group and described a "rapid and efficient synthesis" of bis-MPA dendron and dendrimers.[17] It is worth noting that very recently (February 2015) Malkoch proposed a new method to synthesize bis-MPA dendrimers.[19] It is based on carbonyldiimidazole (CDI) and cesium fluoride (CsF) esterification. The main advantages of the fluoride-promoted esterification method for dendritic growth are to avoid chromatography for purification, and also their extremely fast reaction time. They reported the synthesis of generation 6 bis-MPA dendrimer (22,000 g/mol) within 24h.



Scheme 2.2 – Bis-MPA molecule and its protected derivatives.

To link the dendron to the linear polymer the LDBC coupling strategy in combination with click chemistry, was used. The concept of click chemistry was first introduced by Sharpless[20] in 2001. He defined several criteria for a reaction to be considered a click reaction. It should be "modular, wide in scope and give very high yield" as well as should employ "simple reaction conditions". These criteria apply to a few well known reactions including, thiol-ene, Diels-Alder and copper (I) catalysed alkyne azide cycloaddition (CuAAC), the latter is employed in our studies. The reaction between alkyne

and azide had been known for the past 50 years as the Huigsen or 1,3 dipolar cycloaddition.[21] However, it wasn't used much in synthesis due to poor yields, high temperatures needed and the poor stereoselectivity, as it gave both 1,4 and 1,5 isomers. With the introduction of copper (I) catalyst simultaneously by Forkin and Sharpless[22] and Meldal,[23] the reaction gave high yields, could be carried out at room temperature, had large scope and was stereoselective (1,4 substitution) (Scheme 2.3). In 2004, Fréchet proposed the first CuAAC based dendrimers,[24] and since it has seen rapid use in dendrimer synthesis. Click chemistry has now become an integral part of dendrimer synthesis.[25]



Scheme 2.3 – Copper Catalyzed Alkyne Azide Reaction (CuAAC).

Click chemistry has also been extensively used in LDBC synthesis.[26] Bowman and co-workers used the orthogonality of thiol-Michael addition reaction and CuAAC to prepare LDBC in one pot.[27] The dendron was grown using the thiol-Michael addition to the desired generation, and then PEG diazide was added to the unpurified mixture and reacted with the alkyne at the dendron focal point (Figure 2.1). The orthogonality of click reactions was also exploited by Hvilsted et al., to functionalize one end of poly(ε-caprolactone) with a poly(L-lysine) dendron by CuAAC, and the other end with cholesteryl via thiol-ene, giving a multifunctional LDBC.[28]

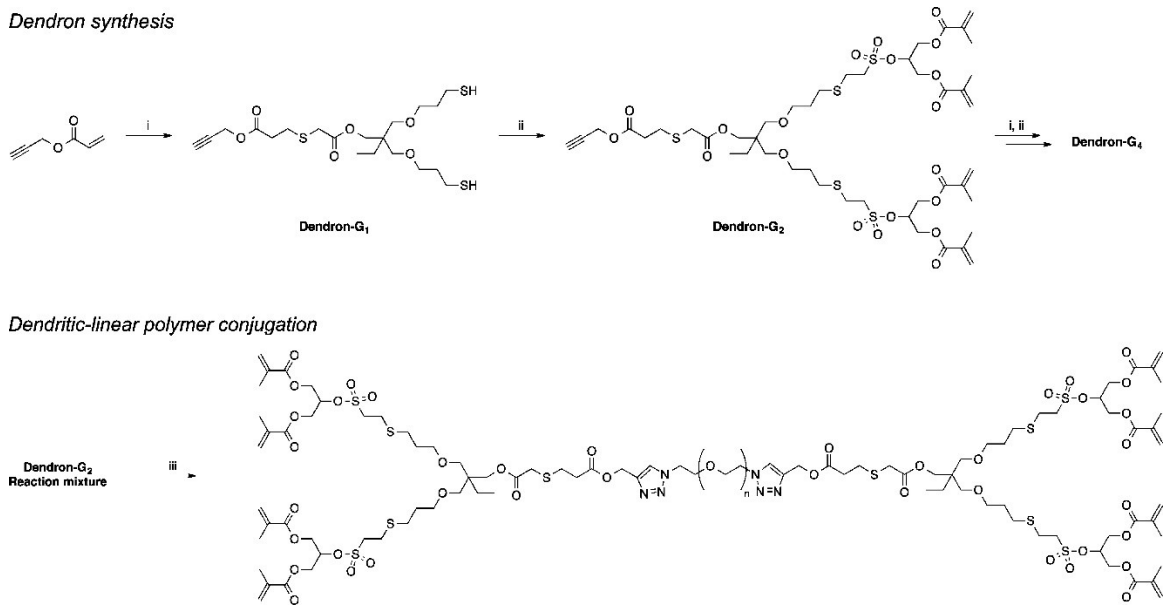


Figure 2.1 –Linear Dendritic Block Copolymer One-Pot Synthesis using Orthogonal Click Chemistry, Reprinted with permission from reference [27]. Copyright 2014 American Chemical Society.

Kempe et al. reported the synthesis of a pH degradable LDBC, made of bis-MPA dendron and poly(2-ethyl-2-oxazoline), by a double-click cascade reaction.[29] The bis-MPA dendron was functionalized with a furan protected maleimide, for Diels-Alder reaction, and the polymer ending with an alkyne group for CuAAC. The cascade reaction took place over 3 days at 115ºC in the presence of the linker, 9-(azidomethyl)anthracene that could undergo both Diels-Alder and CuACC reactions at the same time. The main disadvantage of CuAAC is the use of copper which can be problematic for biological applications if not totally removed from the macromolecule.[30] For this reason, strain promoted alkyne azide cycloaddition is a good alternative to the copper used in CuAAC.[31] Weck et al., used this reaction to functionalize the surface of a dumbbell dendrimer with triethylene glycol.[32]

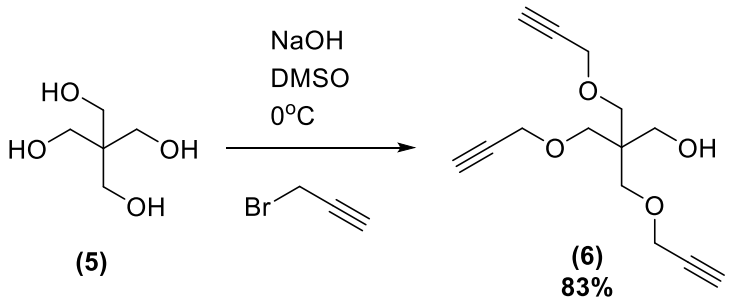
One of the goals of this project is to synthesize a series of pH-sensitive LDBC for drug delivery applications. To achieve this, different generations of bis-MPA dendron were

prepared by divergent synthesis and azido-poly(ethylene glycol) synthesized in varied length through post-polymerization modification. The desired LDBC were prepared by reacting the bis-MPA dendron to a tri-propargylated pentaerythritol core via CuAAC, and the free hydroxyl group of pentaerythritol was then functionalized to introduce PEG. Finally, by including pH-sensitive acetonide group on the dendron surface, stimuli responsive LDBC for drug delivery application were prepared.

2.2 - Results and Discussions

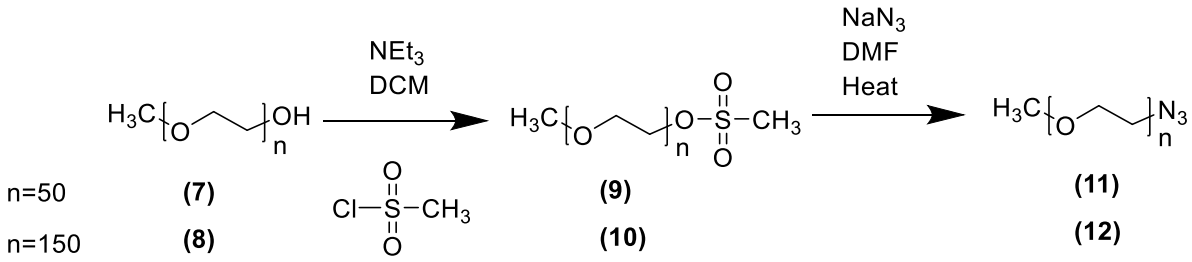
2.2.1 - Monomer synthesis

Pentaerythritol **(5)** was reacted with propargyl bromide via a Williamson etherification (Scheme 2.4). By carefully controlling the reaction conditions, we reacted three of the four hydroxyl groups, however, di and tetra-substituted pentaerythritol were also produced in small amounts by the reaction. To increase the formation of tri-propargylated product the propargyl bromide solution in toluene (80%) needs to be added very slowly to a stirred solution of pentaerythritol at 0°C. Other factors such as the number of equivalents and reaction time seem to a have minor effect on the yield of tri-propargylated pentaerythritol. The product was purified by column chromatography to give the pure tri-propargylated pentaerythritol **(6)**.



Scheme 2.4 – Propargylation of pentaerythritol

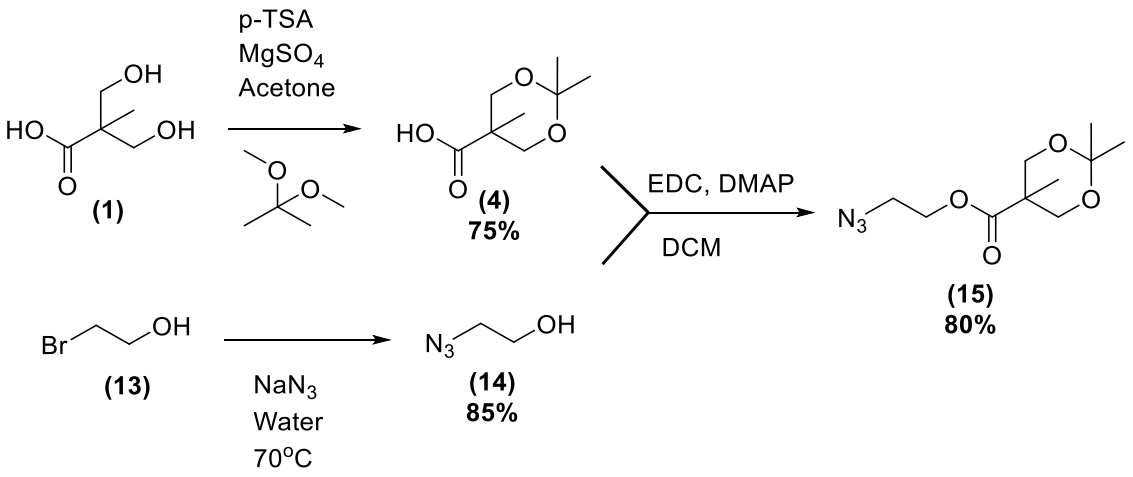
The azido-terminated PEG (N_3PEG) was prepared by post polymerization modification method on two different chain lengths: PEG2000 and 6000 (Scheme 2.5).[33] The hydroxyl group in **7 & 8** was reacted with mesyl chloride in DCM and triethylamine as the base. Since it is a very exothermic reaction, mesyl chloride was slowly added, at 0ºC, to the PEG solution. A simple extraction procedure was used to remove the excess base and mesyl chloride. The products **9 & 10** were analysed by ^{1}H- and ^{13}C-NMR spectroscopy, and subsequently reacted with sodium azide. The azidation was carried out at 70ºC in DMF. Excess sodium azide was removed by extraction and the pure products **11 & 12** analysed by ^{1}H- and ^{13}C-NMR spectroscopy. The disappearance of the mesyl protons is a good indication of the completion of the reaction.



Scheme 2.5 – Synthesis of azido-poly(ethylene glycol) monomethyl ether.

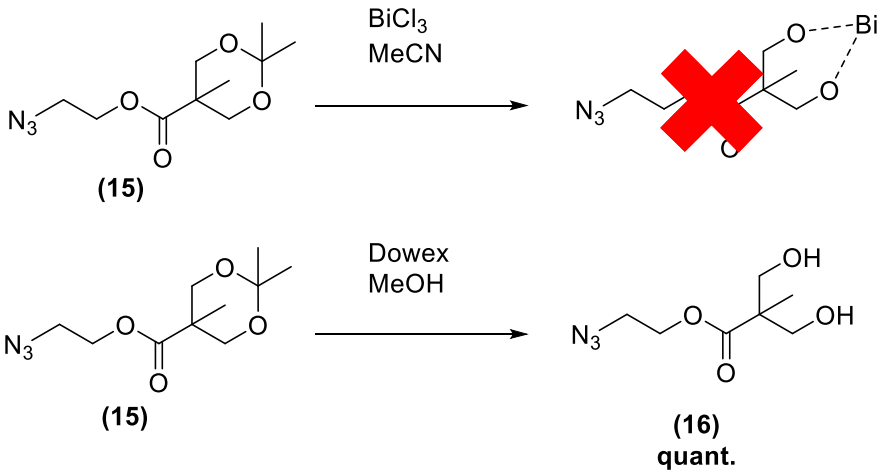
Elaboration of the dendron makes use of 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) monomer **(1)** (Scheme 2.6). The protection of the bis-MPA diol was carried out through a simple acetal formation using acetone and 2,2-dimethoxypropane in dry acidic environment. The protected bis-MPA **(4)** was purified by simple extractions. An azido group was then introduced via a Steglich esterification with protected bis-MPA **(4)** and azidoethanol **(14)**. The latter was prepared from bromoethanol **(13)** by reaction with sodium azide (NaN_3). Using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC·HCl) as coupling reagent and 4-dimethylaminopyridine (DMAP)

as catalyst, the reaction gave, under anhydrous conditions, the azido-bis-MPA **(15)** in 80% yield (Scheme 2.6).



Scheme 2.6 – Synthesis of azido-bis-MPA.

For the deprotection of azido-bis-MPA **(15)** two methods were investigated to remove the ispropylidene group. The first one made use of bismuth trichloride ($BiCl_3$). Although several methanol washes were done to remove bismuth, it was still found to bind to the diols of bis-MPA. To avoid this problem, a second method using Dowex 50W X2, a cationic resin, was employed. The resin was easily removed by filtration and pure deprotected azido-bis-MPA **(16)** was obtained (Scheme 2.7).



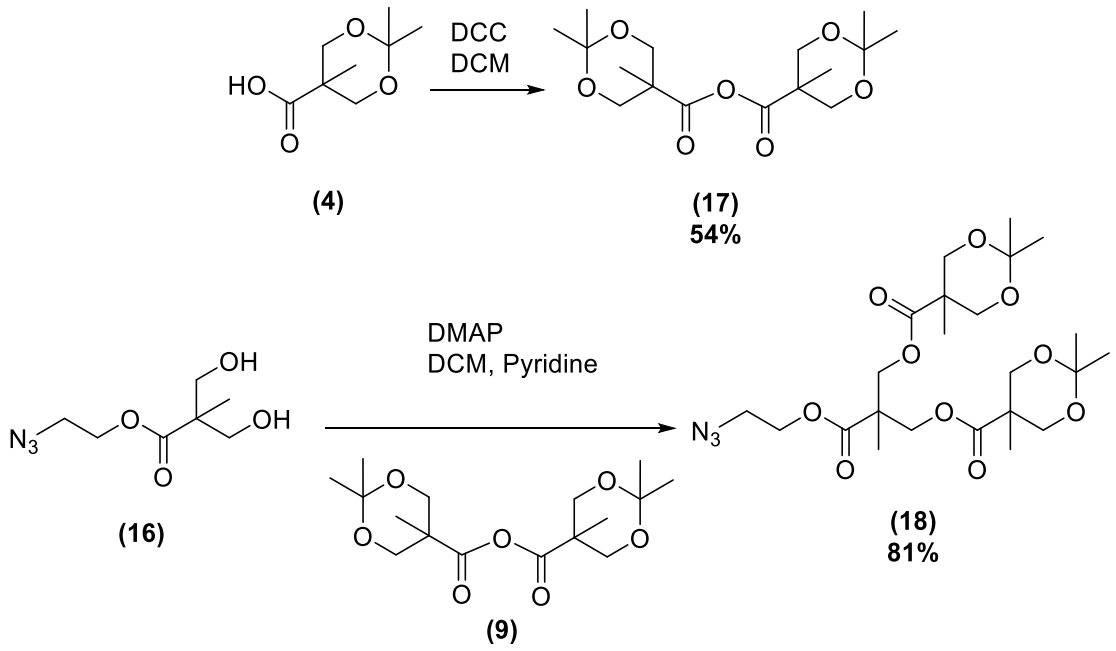
Scheme 2.7 – Deprotection of acetonide-bis-MPA.

2.2.2 - Dendron synthesis

After deprotection, the esterification of another bis-MPA unit can be carried on the diols and several methods were attempted. The first few trials made use of EDC·HCl as the coupling reagent. However, as the reaction proceeded, degradation of the product was observed. The reaction, which was monitored by thin layer chromatography (TLC), yielded small amount of product after extraction, and after further column chromatography no product could be recovered. One of the explanations for this incompatibility between bis-MPA and EDC·HCl probably comes from the hydrochloric acid salt. Esterification reactions produce water that easily hydrolyses the acetonide group in combination with hydrochloric acid. To overcome this problem, other methods were investigated.

N,N'-dicyclohexylcarbodiimide (DCC) is commonly used as a reagent for peptide coupling, and also for esterification.[34] DCC was not the first choice as coupling reagent for many reasons. First, it is extremely toxic and needs to be handled with great care.[35] Second, it is used in combination with pyridine, another very toxic solvent. Finally, it is extremely hard to remove, after completion of the reaction, by conventional methods. DCC, as well

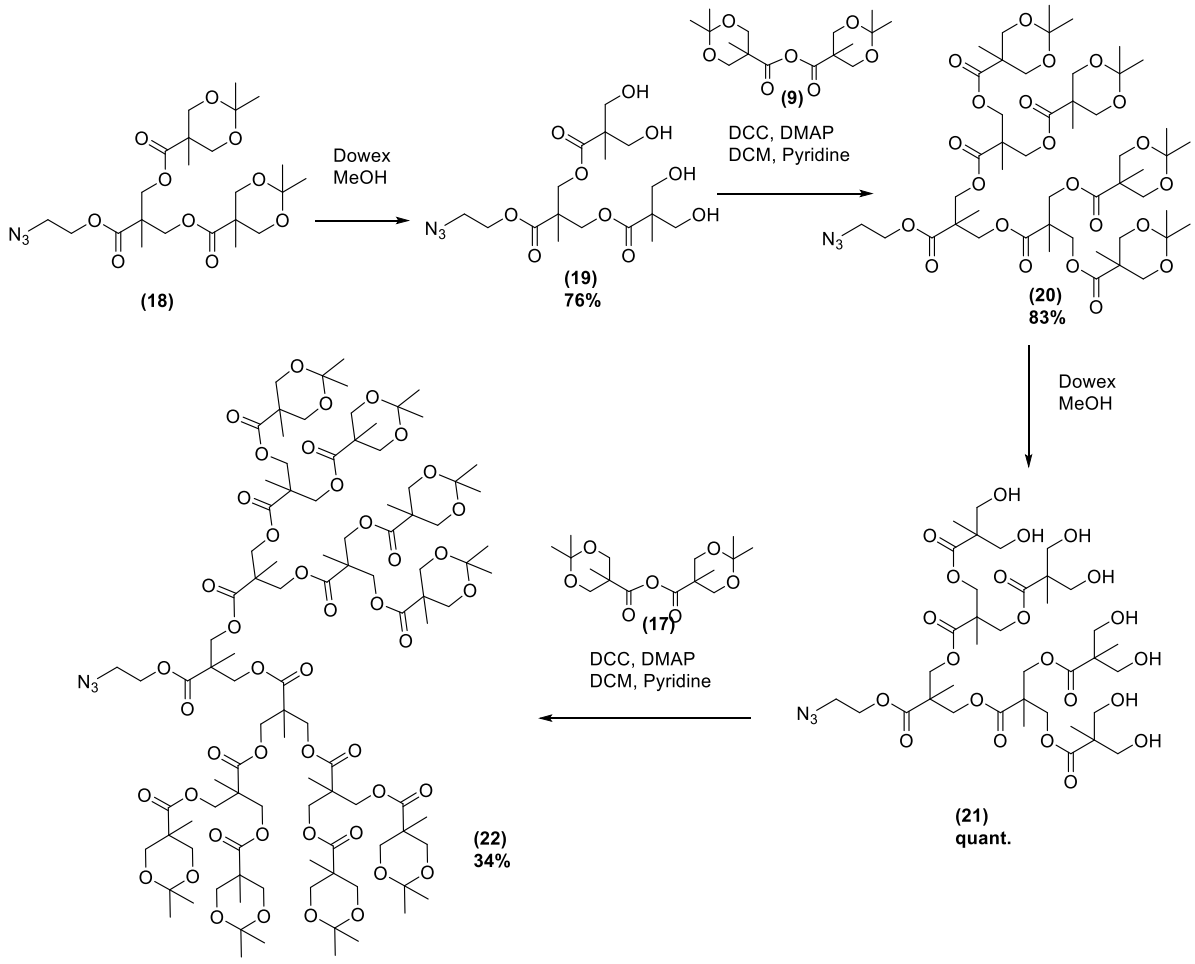
as its by-product dicyclohexylurea, are not soluble in water (cannot be removed by extraction) and are hard to separate using column chromatography. This method being far from ideal, we subsequently noted another methodology developed by Malkoch in 2002.[17] By preparing 2,2-bis(hydroxymethyl)propionic anhydride (bis-MPA anhydride), prior to the esterification, purification becomes less time consuming. On the down side, it still uses DCC as a dehydrating agent for the preparation of the anhydride, and adds an extra step to the synthesis. The anhydride preparation was carried out by mixing DCC and bis-MPA in anhydrous dichloromethane (DCM) and the reaction was monitored by ^{13}C-NMR spectroscopy. The carboxylic acid peak at 180 ppm slowly disappeared and the appearance of an anhydride peak at 169 ppm was noted as the reaction went towards completion. When the carboxylic acid peak totally disappeared the reaction was stopped. The purification was carried out by precipitation of bis-MPA anhydride **(17)** in hexane at -78^{o}C. The esterification step was then performed on azido-bis-MPA **(16)** using DMAP and pyridine in anhydrous DCM under argon, and after column chromatography gave the azido-G1 dendron **(18)** as a transparent oil in 81% yield (Scheme 2.8).



Scheme 2.8 – Synthesis of azido-G1 dendron using bis-MPA anhydride.

The azido-G1 **(18)** dendron was reacted with DOWEX overnight in MeOH at 45°C, to give the deprotected azido-G1 dendron **(19)** in 76% yield (Scheme 2.9). Azido-G2 dendron **(20)** was then prepared by reacting the free hydroxyls group on the surface of the dendron with bis-MPA anhydride at room temperature, under inert atmosphere, for 48h. The product was purified by column chromatography to give the azido-G2 dendron in 83% yield as transparent oil. Finally, the dendron synthesis was elaborated to generation 3. The deprotection of G2 dendron **(20)** using DOWEX in MeOH was quantitative, and gave the deprotected-azido-G2 dendron as a transparent oil **(21)**. The completion of the reaction was monitored by ^{1}H-NMR spectroscopy with the disappearance of acetonide peaks at 1.37 and 1.43 ppm. The azido-G3 dendron **(22)** synthesis was carried out using the bis-MPA anhydride methodology. The reaction was done over 48h at room temperature under argon atmosphere, in the presence of bis-MPA anhydride, DMAP and pyridine in anhydrous DCM. The product was extracted with water, 10% $NaHSO_4$ and 10% Na_2CO_3 to remove

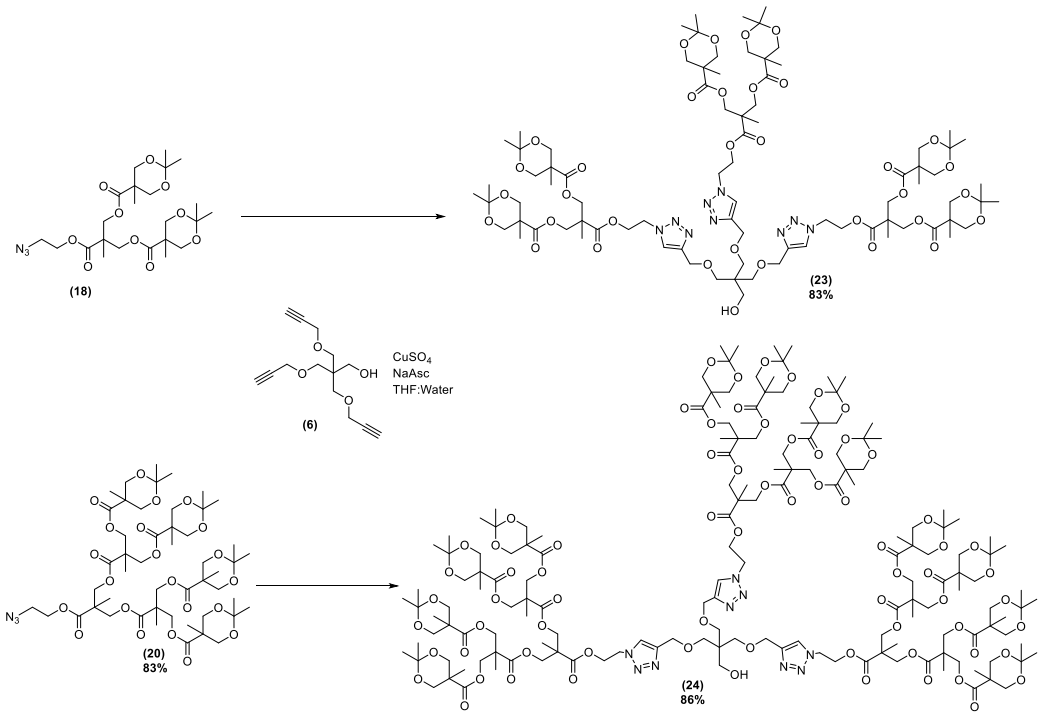
DMAP, pyridine and bis-MPA anhydride, and column chromatography was performed to remove other impurities. Azido-G3 dendron **(22)** was obtained in 34 % yield. The low yield can be partly explained by the loss of the product on the column due to the large molecular weight of the dendron (2170 g/mol).



Scheme 2.9 – Synthesis of G2 and G3 azido-bis-MPA.

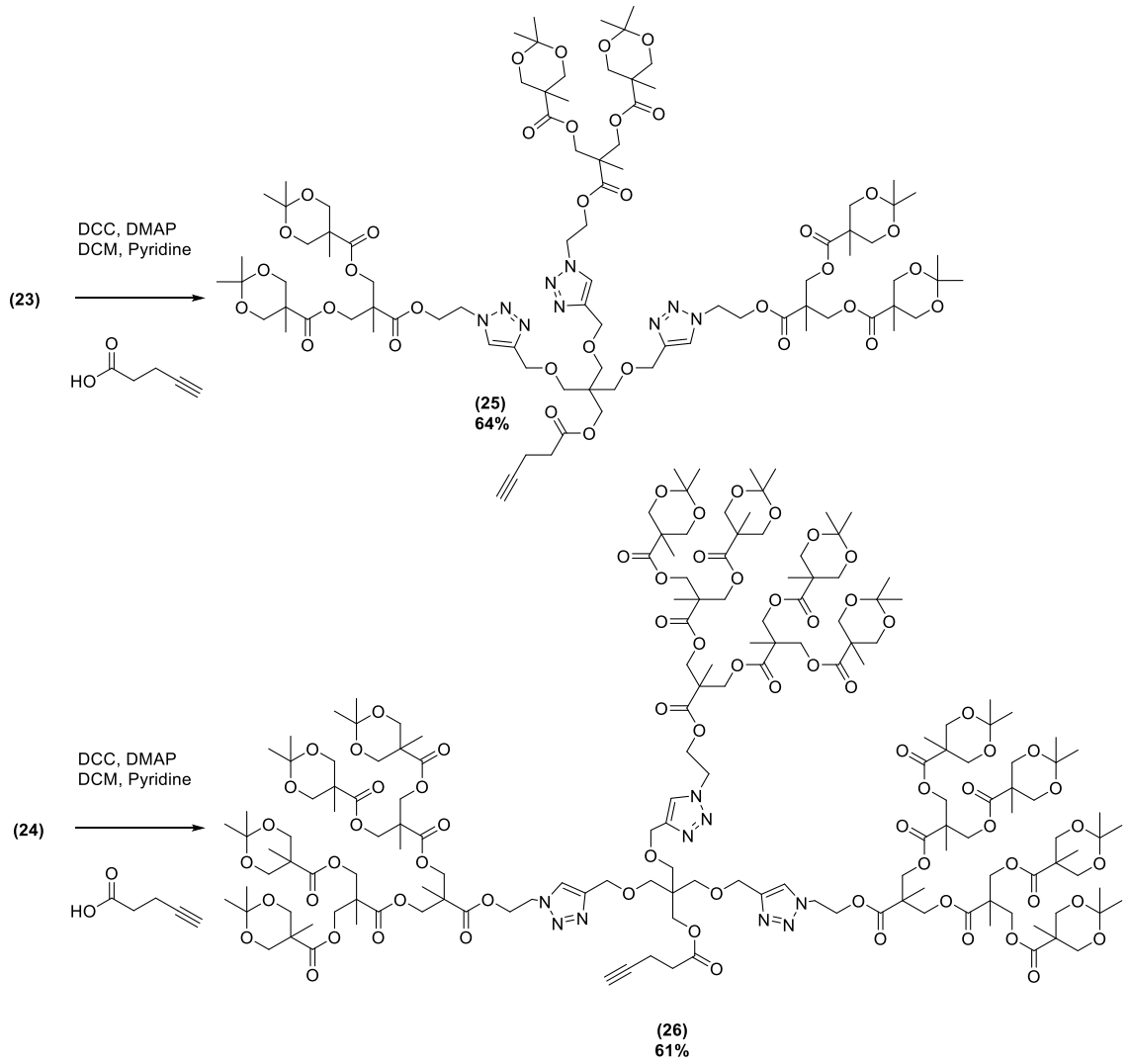
The azido dendrons **18 & 20** were clicked on to pentaerythritol core **(6)** using copper (I) catalyzed alkyne-azide cycloaddition in tetrahydrofuran (THF) and water over 48h at 40ºC (Scheme 2.10). Once the reaction was completed, copper was extracted with a disodium ethylenediaminetetracetic acid solution, and the product was then purified by

column chromatography to give the G1 and G2 dendrimers **(23 & 24)** in 83 and 86% yields respectively.



Scheme 2.10 – Synthesis of G1 and G2 dendron.

Once the bis-MPA dendrons were clicked to pentaerythritol, the free hydroxyl group at the core focal point needed to be functionalized. 4-pentynoic acid was chosen because it contains i) a carboxylic acid that could be easily esterified with the free hydroxyl group, and ii) an alkyne group which could be used for CuAAC. The esterification of 4-pentynoic acid was achieved in anhydrous DCM under argon at room temperature overnight, DCC was used as coupling reagent in combination with DMAP and pyridine. The purification of this reaction was performed by column chromatography and the final yield for G1-pentyne **(25)** and G2-pentyne **(26)** was 64% and 61% respectively (Scheme 2.11). The mediocre yield could likely be due to the fact that the reactive site is small, buried at the core of the dendron and not easily accessible to reactants.

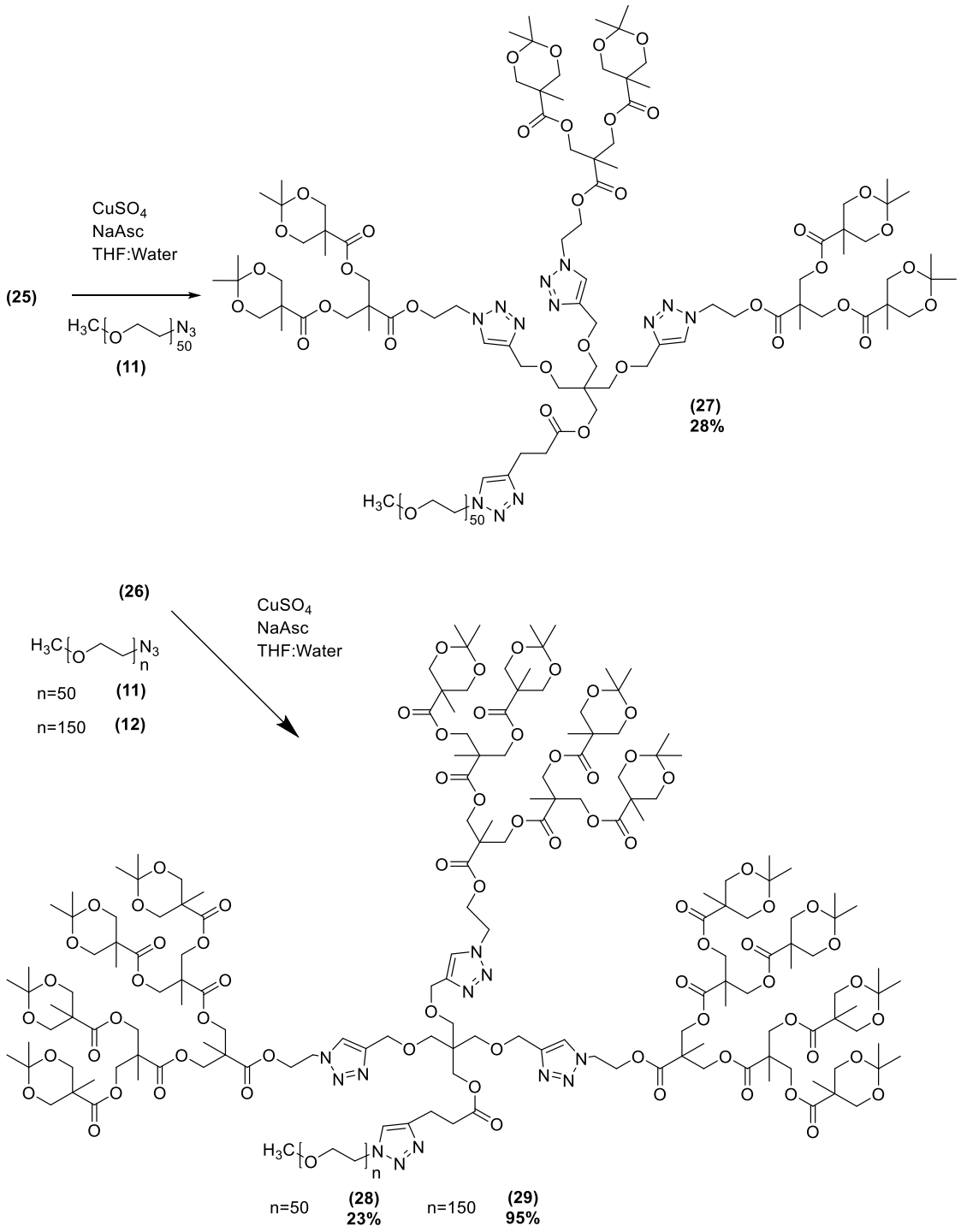


Scheme 2.11 – Synthesis of G1-pentyne and G2-pentyne.

2.2.3 - Linear Dendritic Block Copolymer Synthesis

With an alkyne group at the focal point of the dendrons, N_3PEG **(11 & 12)** can be coupled through CuAAC. The click reaction was carried out by stirring the dendron **(25 & 26)** and the polymer at 40ºC in THF and water in the presence of $CuSO_4$ and sodium ascorbate overnight. Purification of amphiphilic macromolecules can be difficult since

column chromatography and extraction cannot be used for long linear polar chains like polyethylene glycol, that make the macromolecule stick to the column, and soluble in water. In this reaction the copper was removed by stirring Na_2EDTA in DCM overnight. The excess dendron was then separated from LDBCs with multiple precipitations in ether. The dendron was soluble in ether, but not the LDBCs, which could be isolated and gave pure G1PEG2000 **(27)**, G2PEG2000 **(28)** and G2PEG6000 **(29)** in 28, 23 and 95% yield respectively (Scheme 2.12). The lower yield for G1PEG2000 **(27)** and G2PEG2000 **(28)** is due to the shorter chain length of PEG2000 as compared to PEG6000, making it more soluble in ether. The partial solubility of PEG2000 in ether increases the LDBCs solubility in ether, and results in loss of product during the purification process.



Scheme 2.12 – Synthesis of linear dendritic block copolymer.

The LDBCs were fully characterized by gel permeation chromatography (GPC), matrix assisted laser desorption ionization – time of flight (MALDI-TOF) and ^{1}H- and ^{13}C-NMR spectroscopy. Some of the data are summarized in Table 2.1.

Table 2.1 – Summary of GPC and MALDI-TOF results

LDBC	GPC Mn	GPC PD	Theoretical MW	MALDI-TOF
G1PEG2000 **(27)**	4391	1.12	4201.25	4202.99
G2PEG2000 **(28)**	24773	1.12	5503.84	5502.74
G2PEG6000 **(29)**	16291	1.14	10478.8	10476.35
PEG2000 **(11)**	3413	1.07	-	-
PEG6000 **(12)**	12701	1.10	-	-

As shown in Table 2.1, the molecular weights of LDBCs are narrowly distributed with polydispersity in the same range as their linear polymeric segment. The GPC based mass average molecular weight (Mn) data are different from the MALDI-TOF. The standard used for the calibration of the GPC was poly(methyl methacrylate) which has a significantly different molecular structure than the analysed compounds. Furthermore, the dendritic part of LDBC is more globular and interacts differently with the column than a linear macromolecule. These two factors may explain the dissimilarity between the MALDI-TOF and GPC results.

The ^{1}H- and ^{13}C-NMR spectra of LDBCs are similar between generations and chain length, with changes only in the integration of the peaks. G2PEG6000 **(29)** is taken here as an example for a detailed analysis. With 884 protons in the ^{1}H-NMR spectrum, one would expect to see a large peak (3.63 ppm) corresponding to the 600 protons of polyethylene glycol and some smaller, broader and not well resolved peaks corresponding to the

dendritic protons. However, by increasing the acquisition time, the concentration of the sample and relaxation times, it was possible to acquire a well resolved ^{1}H-NMR spectrum. As we can see in Figure 2.2, pentynoic acid protons at 2.68 and 2.98 ppm integrate for two protons each. The protecting group as well as the methyl bis-MPA (1.09-1.44 ppm) also integrate to an overall 126 protons. The newly formed triazole peak (7.52 ppm) is the proof of product formation and integrates perfectly at 1H. The ability to integrate one proton perfectly out of 883 other protons shows how powerful NMR spectroscopy can be for macromolecule characterization.

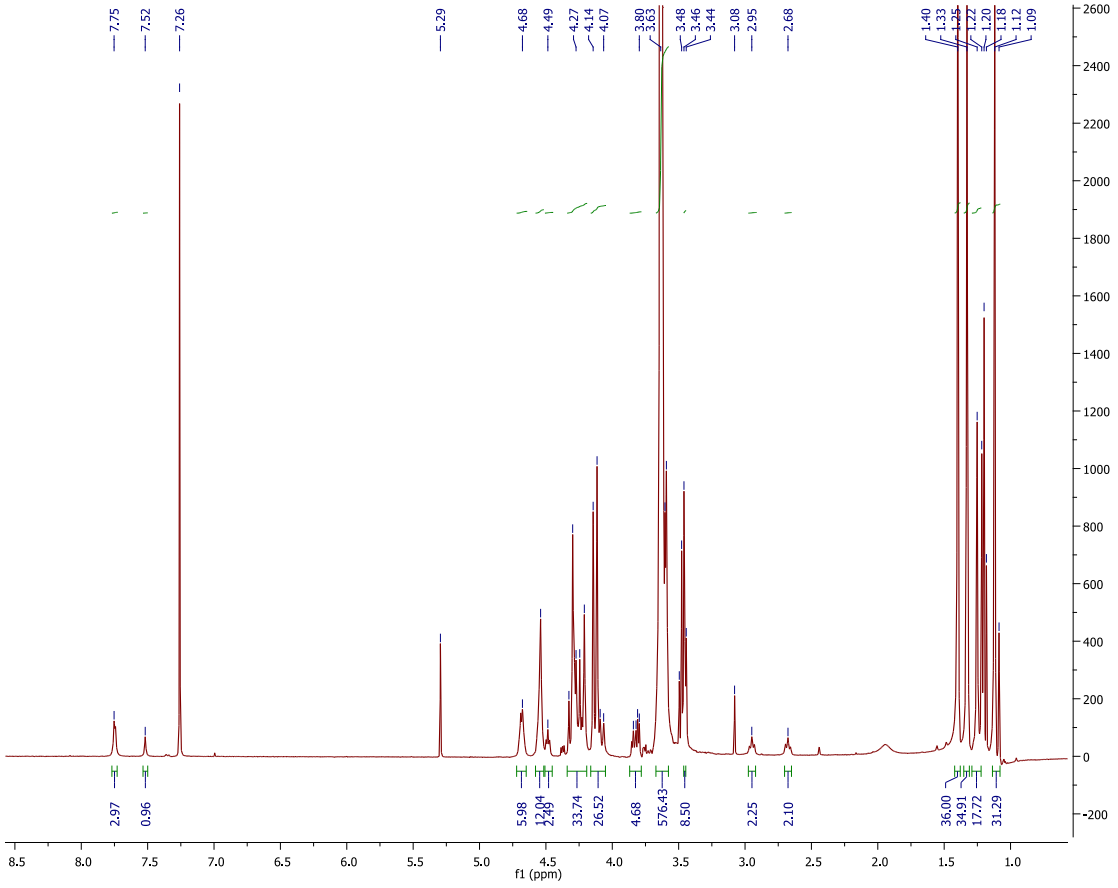
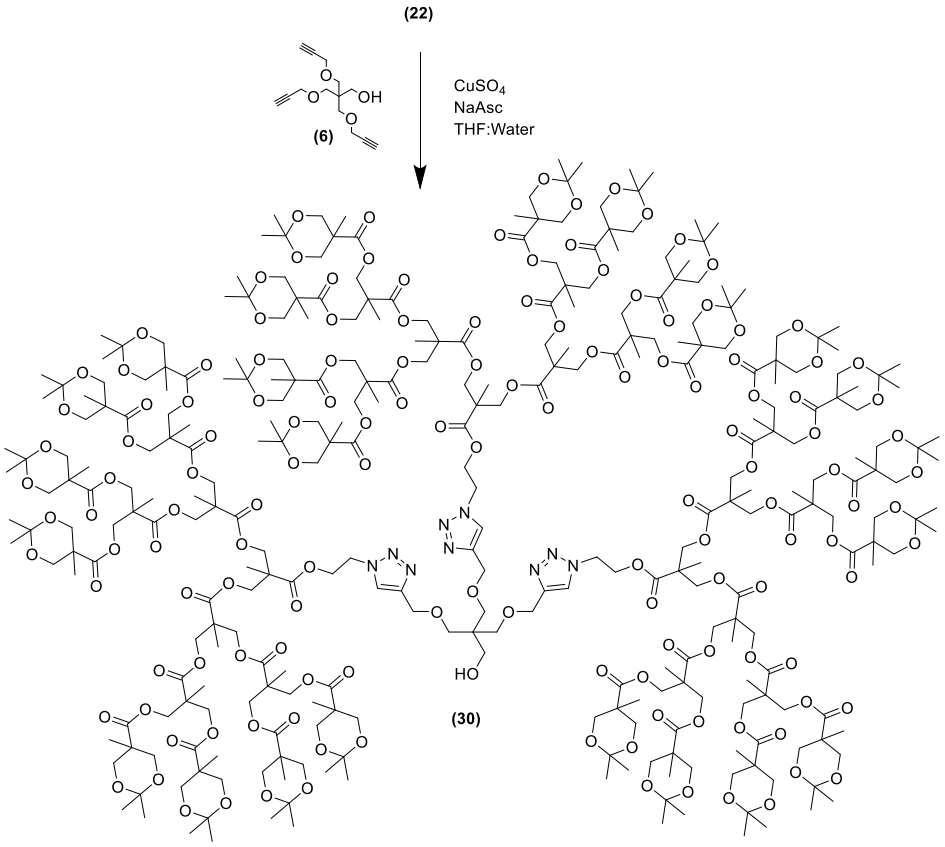


Figure 2.2 – ^{1}H-NMR spectrum of G2PEG6000 in $CDCl_3$.

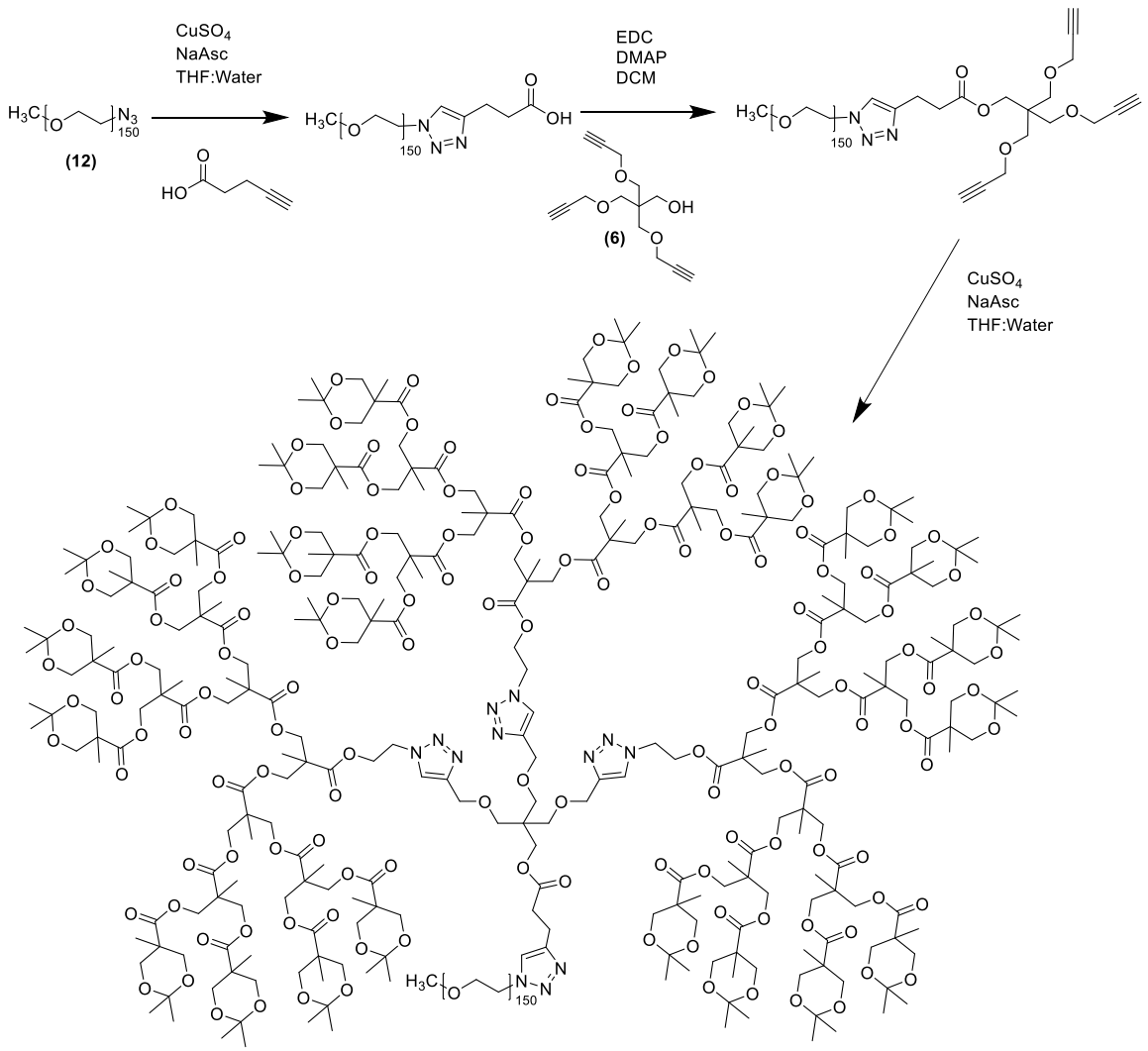
2.2.4 - Generation 3 synthesis

We attempted to click the azido-G3 dendron **(22)** on the pentaerythritol core **(6)** by mixing the dendron, the core and sodium ascorbate in THF at 40ºC for 5 minutes before adding a solution of $CuSO_4$ in water, and leaving the reaction mixture to stir over 72 h at 40ºC (Scheme 2.13). As the reaction occurred, a brown precipitate was formed at the bottom of the round bottom flask. After 72 h the reaction was stopped and the precipitate purified using trituration. It was impossible to analyse the precipitated-solid by solution-state NMR spectroscopy as it was insoluble in most solvents. However, mass spectrometry revealed an incomplete reaction, with the formation of mono-, di- and tri-substituted pentaerythritol **(30)**. Due to insobulity of the product, further purification methods were not tried.



Scheme 2.13 – Synthesis of generation 3 dendrimer.

G3 is still an interesting dendron to explore, and an alternative route to synthesize the corresponding LDBC was designed. Instead of clicking the polymer on the dendron, the dendron could be clicked on the previously functionalized polymer. Poly(ethylene glycol) being soluble in most solvents, the solubility issue previously encountered could be avoided. The major drawback of this method is the difficulty of purification of the functionalized polymer. On a macromolecule larger than 6000 daltons, a tail-modification of 100 daltons is usually not enough to induce significant changes in the physico-chemical properties of the polymer. The separation of the product from the starting material is thus difficult. It is also difficult to ensure quantitative reaction, through spectroscopic technique, as the reactive site is small compare to the overall molecule. However, it is the only possible synthetic pathway to G3 LDBC. Due to lack of time the synthesis was not initiated but Scheme 2.14 presents this alternative route.



Scheme 2.14 – Proposed synthesis of generation 3 LDBC.

2.3 - Conclusions

A series of linear dendritic block copolymer was synthesized using bis-MPA based dendron and poly(ethylene glycol) as the linear counterpart. Copper (I) catalysed alkyne azide cycloaddition as well as Steglich esterification were employed to prepare a series of LDBC with varying dendritic generations and polymeric lengths which were fully characterized using ^{1}H- and ^{13}C-NMR spectroscopy, MS and GPC. We were unable to isolate a pure sample of G3 due to solubility issues, however the new synthetic route

proposed should allow G3 LDBC to be prepared. The LDBC synthesized in this chapter are all amphiphilic, and the following chapter will explore their self-assembly as well as their potential, as pH sensitive drug delivery systems.

2.4 - Experimental

2.4.1 - Materials

The following compounds were purchased and used as received; p-toluene sulfonic acid (TsOH), sodium hydroxide (NaOH), 4-dimethylaminopyridine (DMAP), sodium azide (NaN_3), propargyl bromide 80% solution in toluene, methanesulfonyl chloride (MsCl), bismuth trichloride ($BiCl_3$), 2,2bis(hydroxymethyl)-propionic acid (bis-MPA), DOWEX 50XW4-200 ion exchange resin, sodium ascorbate, copper(II) sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$), pentaerythritol, poly(ethylene glycol) methyl ether 2000 MW, and ethylenediamine tetraacetate disodium salt (Na_2EDTA) from Sigma Aldrich (USA and Canada), poly(ethylene glycol) methyl ether 6000 MW from J.T Baker (USA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride salt (EDC·HCl) from Chem Impex International (USA) and n,n'-dicylcohexylcarbodiimide (DCC) from Alfa Aesar (USA). (USA). Polyvinylidene Fluoride (PVDF) Syringe filters of pore size 0.45 μm and 17 mm diameter were purchased from Sterlitech (USA).

The solvents dimethyl sulfoxide (DMSO), triethyl amine (NEt_3), pyridine, methanol (MeOH), acetonitrile (MeCN), tetrahydrofuran (THF), acetone, dimethylformamide (DMF), chloroform ($CHCl_3$), dichloromethane (DCM), as well as the drying agent magnesium sulfate ($MgSO_4$) and sodium sulfate (Na_2SO_4) were purchased from Fisher scientific and ACP Chemicals and used as received. Dry solvents were

obtained from dry solvent system. Milli-Q Ultrapure water was doubly distilled by reverse osmosis though a Millipore RiOS8, followed by filtration through a Milli-Q Academic A10 filtration unit prior to use.

NMR spectral acquisitions were carried out on 400 MHz Mercury (Varian) instruments and operated using VNMRJ 2.2D (Chempack 5) and VNMRJ 2.3A (Chempack 5) software, as well as on an AV 400 and 500 MHz (Bruker) using a 5 mm Smart Probe. The chemical shifts in ppm are reported relative to tetramethylsilane (TMS) as an internal standard for ^{1}H-, and ^{13}C- NMR spectra.

The number-average molecular weight (M_n) and the molecular weight distribution (M_w/M_n) were determined using GPC (Waters Breeze) with THF as the mobile phase at 0.3 mL.min^{-1}. The GPC was equipped with a guard column and 3 Waters Styragel HR columns: HR1 with molecular weight measurement range of 10^2–5×10^3 g mol^{-1}, HR2 with molecular weight measurement range of 5×10^2–2×10^4 g mol^{-1}, and HR4 with molecular weight measurement range of 5×10^3–6×10^5 g mol^{-1}. The columns were heated to 40 °C during the analysis. The molecular weights were determined by calibration with linear narrow molecular weight distribution poly(methyl methacrylate), and the GPC was equipped with a differential refractive index (RI 2410) detectors.

Mass spectra analyses (HRMS, ESI) were performed and analysed on an Exactive Plus Orbitrap-API (Thermo Scientific) high resolution mass spectrometer and on MALDI Autoflex III – TOF (Brucker).

2.4.2 - Methods

The following molecules were synthesized using an elaboration and modification of the procedures described in references 37 **(6)**, 15 **(4, 17)**, 34 **(9, 10, 11, 12, 15, 16, 18, 19),** 38 **(14)**.

Synthesis of tripropargyl-pentaerythritol **(6)**

Pentaerythritol **(5)** (2.00 g, 0.015 mol) was dissolved in DMSO (15 mL) and a solution of NaOH (3.20 g, 0.080 mol) in water (8 mL) was added to it. After 30 minutes stirring at room temperature, a solution of propargyl bromide (9.52 g, 11.9 mL, 0.080mol) in toluene (80%) was added drop wise to the reaction mixture. The reaction was left stirring at room temperature overnight. Once the reaction was completed, water was added to the mixture and extracted with diethyl ether. The organic layers were isolated, combined, washed with water (3x) and brine (3x) and dried with sodium sulfate. The ether was removed to yield an orange oil which was purified by column chromatography (75% hexane 25% ether to 100% ether) to give the product as a yellow oil (3.15 g, 0.013 mol, 86% yield). ^{1}H-NMR (400MHz, $CDCl_3$): δ = 2.35 (s, HO-CH_2-C, 1H) 2.41 (t, CH-C-CH_2-, 3H), 3.54 (s, C-CH_2-OH, 2H) 3.67 (s, C-CH_2-O-, 6H), 4.12 (d, CH-C-CH_2-, 6H) ppm. ^{13}C-NMR (75MHz, $CDCl_3$) δ = 44.6, 58.7, 65.0, 70.1, 74.5, 79.6 ppm.

Synthesis of 2,2,5-trimethyl-1,3-dioxane-5-carboxylic acid **(4)**

Para-toluenesulfonic acid (4.8 g, 0.024 mol) was added to a stirred solution of 2,2-bis(hydroxymethyl)propionoic acid **(1)** (60.0 g, 0.448 mol) in acetone (100mL), under argon. 2,2-dimethoxypropane (72.0 g, 0.692 mol) and magnesium sulfate (7.2 g, 0.060

mol) were then added to the flask. The reaction mixture was left stirring under argon for 24h at room temperature. The reaction was quenched with a 4M ammonia solution in dioxane (39.24 mL) and stir for an additional 30 minutes. The insoluble solid was then filtered, and solvents evaporated. The solid mixture was then dissolved in DCM and extracted with water (x3). The organic layer was dried with Na_2SO_4 and the solvent evaporated to yield the product as a white powder (58.5 g, 0.0524mol, 75% yield). ^{1}H-NMR (400MHz, $CDCl_3$): δ = 1.24 (s, -CO-C-C$\underline{H_3}$. 3H), 1.44 (s, -O-C-C$\underline{H_3}$, 3H), 1.47 (s, -O-C-C$\underline{H_3}$, 3H), 3.70 (d, -O-C$\underline{H_2}$-C-CO-, 2H), 4.21 (d, -O-C$\underline{H_2}$-C-CO-, 2H) ppm.

Synthesis of mesyl-terminated polyethylene glycol 2000 **(9)**

To a mixture of poly(ethylene glycol) monomethyl ether (3.00 g, 1.5 mmol) in DCM (250 mL) at 0^{o}C was added triethylamine (1.51 g, 2.1 mL, 15.0 mmol) and methanesulfonyl chloride (1.71 g, 1.20 mL, 15.0 mmol). The reaction was let to stir at room temperature overnight. The reaction was then extracted with 1M HCl (x3), 1M NaOH (x3) and brine (x6). The organic phase was dried with Na_2SO_4 and the solvent removed under reduce pressure. The product obtained was a white waxy solid. ^{1}H-NMR (500MHz, $CDCl_3$): δ = 3.06 (s, -O-SO_2-C$\underline{H_3}$. 3H), 3.34 (s, -CH_2-O-C$\underline{H_3}$, 3H), 3.49-3.53 (t, H_3C-O-C$\underline{H_2}$-CH_2-, 2H), 3.56-3.64 (br, -O-C$\underline{H_2}$-C$\underline{H_2}$-O, 200H), 3.72-3.75 (t, -CH_2-C$\underline{H_2}$-O-SO_2-, 2H) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 37.7, 59.0, 69.3, 70.5, 71.9 ppm.

Synthesis of azido polyethylene glycol 2000 **(11)**

To a solution of mesyl-terminated polyethylene glycol (3.0 g, 1.5 mmol) in dimethylformamide (5 mL) was added sodium azide (1.0 g, 15.0 mmol). The mixture was

stirred overnight at 50ºC. The reaction was stopped by addition of DCM to the room temperature mixture. The organic phase was then extracted with water (x4) and brine (x5), dried with Na_2SO_4 and removed *in vacuo*. The azido polyethylene glycol obtained was a white waxy solid (2.059 g, 1.03 mmol, 69% yield over two reactions, mesylation and azidation). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 3.28 (s, -CH_2-O-C$\underline{H_3}$, 3H), 3.37 (t, -CH_2-C$\underline{H_2}$-N_3, 2H), 3.49-3.53 (t, H_3C-O-C$\underline{H_2}$-C$\underline{H_2}$-, 4H), 3.56-3.64 (br, -O-C$\underline{H_2}$-C$\underline{H_2}$-O, 200H) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 50.6, 58.9, 69.9, 70.6, 71.8 ppm.

Synthesis of mesyl-terminated polyethylene glycol 6000 **(10)**

To a mixture of polyethylene glycol (2.00 g, 0.33 mmol) in DCM (70 mL) at 0ºC was added triethylamine (0.34 g, 0.47 mL, 3.6 mmol) and methanesulfonyl chloride (0.38 g, 0.26 mL, 3.3 mmol). The reaction was let to stir at room temperature overnight. The reaction was then extracted with 1M $NaHSO_4$ (x3), 1M $NaHCO_3$ (x3) and brine (x3). The organic phase was dried with Na_2SO_4 and the solvent removed under reduce pressure. The product obtained was a white crystalline solid.). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 3.06 (s, -O-SO_2-C$\underline{H_3}$. 3H), 3.34 (s, -CH_2-O-C$\underline{H_3}$, 3H), 3.49-3.53 (t, H_3C-O-C$\underline{H_2}$-CH_2-, 2H), 3.56-3.64 (br, -O-C$\underline{H_2}$-C$\underline{H_2}$-O, 600H), 3.72-3.75 (t, -CH_2-C$\underline{H_2}$-O-SO_2-, 2H) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 37.7, 53.5, 69.3, 70.6 ppm.

Synthesis of azido polyethylene glycol 6000 **(12)**

To a solution of mesyl-terminated polyethylene glycol (2.0 g, 0.33 mmol) in dimethylformamide (5 mL) was added sodium azide (0.2 g, 3.08 mmol). The mixture was stirred overnight at 50ºC. The reaction was stopped by addition of DCM to the room

temperature mixture. The organic phase was then extracted with water (x3) and brine (x6), dried with Na_2SO_4 and removed *in vacuo*. The azido polyethylene glycol obtained was a white crystalline solid. (1.857 g, 0.31 mmol, 94% yield over two reactions, mesylation and azidation). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 3.28 (s, -CH_2-O-C$\underline{H_3}$, 3H), 3.37 (t, -C$\underline{H_2}$-C$\underline{H_2}$-N_3, 4H), 3.56-3.64 (br, -O-C$\underline{H_2}$-C$\underline{H_2}$-O, 600H), 3.73 (t, H_3C-O-C$\underline{H_2}$-C$\underline{H_2}$-, 4H) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 50.6, 70.0, 70.6 ppm.

Synthesis of Azidoethanol **(14)**

A mixture of bromoethanol **(13)** (10.00 g, 0.081 mol) and sodium azide (18.00 g, 0.287 mol) in water (15 mL) was stirred overnight at 70°C. The reaction mixture was then extracted with diethyl ether, the organic phase was dried with Na_2SO_4. The solvent was then evaporated to yield the product as a yellow oil (6.00 g, 0.069 mol, 85% yield). ^{1}H-NMR (400MHz, $CDCl_3$): δ = 3.39 (t, 2H), 3.73 (q, 2H) ppm.

Synthesis of 2-azidoethyl 2,2,5-trimethyl-1,3-dioxane-5-carboxylate **(15)**

A solution of azidoethanol **(14)** (2.50 g, 0.029 mol), 2,2,5-trimethyl-1,3-dioxane-5-carboxylic acid **(4)** (6.07 g, 0.035 mol) and 4-dimethylaminopyridine (DMAP) (1.77 g, 0.145 mol) in anhydrous DCM (25 mL) was left stirring, under argon, for 10 minutes. 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) (5.43 g, 0.035 mol) was then added to the reaction mixture and stirred at room temperature overnight. The reaction mixture was dissolved with more DCM and extracted in water (x3). The organic phase was dried with Na_2SO_4 and the solvent evaporated to yield a residue that was

purified by column chromatography (1:7 EtOAc:Hexane). The final product was obtained as a white solid (4.11 g, 0.017 mol, 80% yield). ^{1}H-NMR (400MHz, $CDCl_3$): δ = 1.21 (s, -CO-C-C$\underline{H}_3$, 3H), 1.39 (s, -O-C-C$\underline{H}_3$, 3H), 1.44 (s, -O-C-C$\underline{H}_3$, 3H), 3.49 (t, N_3-C$\underline{H}_2$-CH_2-, 2H), 3.68 (d, -O-C$\underline{H}_2$-C-CO-, 2H), 4.21 (d, -O-C$\underline{H}_2$-C-CO-, 2H), 4.33 (t, N_3-CH_2-C$\underline{H}_2$-CO, 2H) ppm.

Synthesis of deprotected azido-bis-MPA **(16)**

DOWEX 50W-X2 (2.00 g) was added to a solution of azido-bis-MPA **(15)** (4.11 g, 0.017 mol) in methanol (50 mL). The mixture was heated at 45ºC overnight. The resin was then filtered off and wash thoroughly with methanol. The solvent was evaporated to yield **(16)** as a transparent oil (3.45 g, 0.017 mol, quantitative). ^{1}H-NMR (400MHz, MeOD): δ = 1.20 (s, -CH_2-C-C$\underline{H}_3$, 3H), 3.51 (t, N_3-C$\underline{H}_2$-CH_2-, 2H), 3.66 (d, HO-C$\underline{H}_2$-C-CO-, 2H), 3.73 (d, HO-C$\underline{H}_2$-C-CO-, 2H), 4.28 (t, N_3-CH_2-C$\underline{H}_2$-, 2H) ppm. ^{13}C NMR (75MHz, MeOD): δ = 18.5, 42.0, 49.8, 63.6, 65.9, 174.0 ppm.

Synthesis of bis-MPA anhydride **(17)**

DCC (10.50 g, 0.051 mol) was added to a solution of bis-MPA **(4)** (17.74 g, 0.102 mol) in anhydrous DCM (50 mL). The reaction was let to stir at room temperature under argon for 48h. Once the reaction was completed, the precipitate was filtered off. The DCM was evaporated under reduced pressure to obtain a yellowish oil residue. Hexane was added to the residue stirred until a solid formed. The solution was then cooled at -78ºC for 1 h and vacuum filtered through a glass filter. The process was repeated two more times to afford **(17)** as white crystals (18.18 g, 0.055 mol, 54% yield). ^{1}H-NMR (400MHz,

$CDCl_3$): δ = 1.26 (s, -CO-C-C$\underline{H_3}$. 6H), 1.41(s, -O-C-C$\underline{H_3}$, 6H), 1.45 (s, -O-C-C$\underline{H_3}$, 6H), 3.70 (t, -O-C$\underline{H_2}$-C-CO-, 4H), 4.22 (d, -O-C$\underline{H_2}$-C-CO-, 4H) ppm. ^{13}C-NMR (75MHz, $CDCl_3$): δ = 17.6, 21.8, 25.4, 43.6, 65.8, 98.4, 169.7 ppm.

Synthesis of G1 dendron **(18)**

To a mixture of deprotected azido-bis-MPA **(16)** (1.959 g, 0.00965 mol) and DMAP (0.353 g, 0.0029 mol) in pyridine (7.620 g, 7.76 mL, 0.0965 mol) was added a solution of bis-MPA anhydride **(17)** (8.280 g, 0.0251 mol) in DCM (25 mL). The reaction was let to stir at room temperature overnight. Once the reaction was completed, 2 mL of water was added to quench the excess anhydride. Then the reaction was diluted in DCM and extracted with water (3x), 10% $NaHSO_4$ (3x), 10% Na_2CO_3 (3x) and brine (1x). The organic layer was isolated and dried with Na_2SO_4 and concentrated. The residue obtained was purified by column chromatography (1:1 hexane:EtOAc) to give a transparent sticky oil **(18)** (4.006 g, 0.0778 mol, 81% yield). ^{1}H-NMR (400MHz, $CDCl_3$): δ = 1.16 (s, -O-C-C$\underline{H_3}$, 6H), 1.34 (s, -CO-C-C$\underline{H_3}$, 3H), 1.37 (s, -CO-C-C$\underline{H_3}$, 6H), 1.43 (s, -O-C-C$\underline{H_3}$, 6H), 3.51 (t, N_3-CH_2-CH_2-, 2H), 3.64 (d, -O-C$\underline{H_2}$-C-CO-, 4H), 4.17 (d, -O-C$\underline{H_2}$-C-CO-, 4H), 4.33 (t, N_3-CH_2-CH_2-, 2H), 4.36 (d, -O-C$\underline{H_2}$-C-CO-, 4H), ppm.

Synthesis of deprotected G1 dendron **(19)**

DOWEX 50W-X2 (0.700 g) was added to a solution of G1dendron **(18)** (1.558 g, 0.00303 mol) in methanol (20 mL). The mixture was heated at 45ºC overnight. The resin was then filtered off and wash thoroughly with methanol. The solvent was evaporated to yield **(19)** as a transparent oil (1.004 g, 2.29 mmol, 76 % yield). ^{1}H-NMR (500MHz,

$CDCl_3$): δ = 1.08 (s, -CH_2-C-C$\underline{H_3}$, 6H), 1.35 (s, -CH_2-C-C$\underline{H_3}$, 3H), 3.51 (t, N_3-C$\underline{H_2}$-CH_2-, 2H), 3.70 (d, HO-C$\underline{H_2}$-C-CO-, 4H), 3.83 (d, HO-C$\underline{H_2}$-C-CO-, 4H), 4.32 (m, -O-C$\underline{H_2}$-C-CO-, 4H), 4.44 (t, N_3-CH_2-C$\underline{H_2}$-, 2H) ppm. ^{13}C-NMR (125MHz, MeOD): δ = 17.1, 17.98, 42.0, 46.5, 53.45, 63.6, 64.8, 67.3, 172.8, 175.0 ppm.

Synthesis of G2 dendron **(20)**

To a mixture of deprotected G1 dendron **(19)** (1.004 g, 0.0023 mol) and DMAP (0.168 g, 0.0014 mol) in pyridine (3.618 g, 3.70 mL, 0.0458 mol) was added a solution of bismpa anhydride **(17)** (3.927 g, 0.0119 mol) in DCM (10 mL). The reaction was let to stir at room temperature for 48h. Once the reaction was completed, 1 mL of water was added to quench the excess anhydride. Then the reaction was diluted in DCM and extracted with water (3x), 10% $NaHSO_4$ (3x), 10% Na_2CO_3 (3x) and brine (1x). The organic layer was isolated and dried with Na_2SO_4 and concentrated. The residue obtained was purified by column chromatography (1:2 hexane:EtOAc) to give a transparent sticky oil **(20)** (2.020 g, 0.0019 mol, 83% yield). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 1.17 (s, -O-C-C$\underline{H_3}$, 12H), 1.26 (s, -CO-C-C$\underline{H_3}$, 6H), 1.30 (s, -CO-C-C$\underline{H_3}$, 3H), 1.37 (s, -CO-C-C$\underline{H_3}$, 12H), 1.43 (s, -O-C-C$\underline{H_3}$, 12H), 3.54 (t, N_3-C$\underline{H_2}$-CH_2-, 2H), 3.65 (d, -O-C$\underline{H_2}$-C-CO-, 8H), 4.17 (d, -O-C$\underline{H_2}$-C-CO-, 8H), 4.26-4.34 (m, N_3-CH_2-C$\underline{H_2}$-CO-, -O-C$\underline{H_2}$-C-CO-, 14H) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 14.2, 17.5, 18.5, 25.1, 42.0, 46.7, 49.6, 60.3, 64.1, 64.9, 66.0, 98.1, 171.1, 171.9, 173.5 ppm. HRMS (ESI) m/z: [M+Na] + Calculated for $C_{49}H_{77}O_{22}N_3Na$ 1082.4891; Found 1082.4886

Synthesis of deprotected G2 dendron **(21)**

A teaspoon of DOWEX 50W-X2 was added to a solution of G2 dendron **(20)** (760 mg, 0.717 mmol) in methanol (10 mL). The mixture was heated at 45ºC overnight. The resin was then filtered off and wash thoroughly with methanol. The solvent was evaporated to yield **(21)** as a transparent oil (645 mg, 0.717 mmol, quantitative). ^{1}H-NMR (500MHz, MeOD): δ = 1.17 (s, -CH_2-C-C$\underline{H}_3$, 12H), 1.32 (s, -CH_2-C-C$\underline{H}_3$, 6H), 1.35 (s, -CH_2-C-C$\underline{H}_3$, 3H), 3.51 (t, N_3-C$\underline{H}_2$-CH_2-, 2H), 3.57-3.63 (m, HO-C$\underline{H}_2$-C-CO-, HO-C$\underline{H}_2$-C-CO-, 22H), 4.28-4.35 (m, -O-C$\underline{H}_2$-C-CO-, N_3-CH_2-C$\underline{H}_2$-, 16H) ppm. ^{13}C-NMR (125MHz, MeOD): δ = 15.9, 46.5, 50.4, 64.4, 77.9, 174.9 ppm.

Synthesis of G3 dendron **(22)**

To a mixture of deprotected G2 dendron **(21)** (645 mg, 0.72 mmol) and DMAP (131 mg, 1.08 mmol) in pyridine (2.260 g, 2.30 mL, 28.68 mmol) was added a solution of bis-MPA anhydride **(17)** (2.460 g, 7.46 mmol) in DCM (5 mL). The reaction was let to stir at room temperature for 48h. Once the reaction was completed, 1 mL of water was added to quench the excess anhydride. Then the reaction was diluted in DCM and extracted with water (3x), 10% $NaHSO_4$ (3x), 10% Na_2CO_3 (3x) and brine (1x). The organic layer was isolated and dried with Na_2SO_4 and concentrated. The residue obtained was purified by column chromatography (1:2 hexane:EtOAc) to give a transparent sticky oil **(22)** (520 mg, 0.242 mol, 34% yield). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 1.16 (s, -O-C-C$\underline{H}_3$, 24H), 1.28 (s, -CO-C-C$\underline{H}_3$, 18H), 1.30 (s, -CO-C-C$\underline{H}_3$, 3H), 1.37 (s, -CO-C-C$\underline{H}_3$, 24H), 1.43 (s, -O-C-C$\underline{H}_3$, 24H), 3.54 (t, N_3-C$\underline{H}_2$-CH_2-, 2H), 3.65 (d, -O-C$\underline{H}_2$-C-CO-, 16H),

4.17 (d, -O-C$\underline{H_2}$-C-CO-, 16H), 4.27-4.34 (m, N_3-CH_2-C$\underline{H_2}$-CO-, -O-C$\underline{H_2}$-C-CO-, 30H) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 17.1, 17.5, 18.5, 25.1, 42.0, 46.7, 46.8, 49.6, 64.2, 64.8, 66.0, 98.1, 171.1, 171.8, 173.5 ppm. HRMS (ESI) m/z: [M+Na] + Calculated for $C_{101}H_{994}O_{46}N_3Na$ 2170.994; Found 2170.990

Synthesis of G1 **(23)**

To a mixture of G1 dendron **(18)** (3.61 g, 0.0070 mol) and tripropargyl-pentaerythritol **(6)** (0.486 g, 0.0019 mol) in THF (30 mL) was added sodium ascorbate (0.225 g, 0.00114 mol). After stirring for 5 minutes a solution of aqueous copper sulfate (142 mg, 0.00057 mol in 3 mL of water) was added to the stirring mixture. The reaction was warmed at 40ºC and stirred overnight under argon. Once the reaction was completed the organic solvent was removed *in vacuo*. The mixture was dissolved in DCM and extracted with water (x3), EDTA solution (x3) and brine (x1). The organic phase was dried with Na_2SO_4 and the product concentrated. The residue was purified by column chromatography (100% EtOAc to 5% MeOH in DCM) to give a sticky white foam **(23)** (2.830 g, 0.00158 mol, 83% yield). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 1.23 (s, -O-C-C$\underline{H_3}$, 18H), 1.26 (s, -CO-C-C$\underline{H_3}$, 9H), 1.32 (s, -CO-C-C$\underline{H_3}$, 18H), 1.40 (s, -O-C-C$\underline{H_3}$, 18H), 3.51 (t, N-C$\underline{H_2}$-CH_2-, 6H), 3.60 (d, -O-C$\underline{H_2}$-C-CO-, 12H), 4.12 (d, -O-C$\underline{H_2}$-C-CO-, HO-C$\underline{H_2}$-C-, 14H), 4.30 (dd, -O-C$\underline{H_2}$-C-CO-, 12H), 4.59 (m, -N-CH_2-C$\underline{H_2}$-CO-,-O-C$\underline{H_2}$-C-CH-N-, -O-C$\underline{H_2}$-C- 18H), 7.73 (s, 3H, -O-CH_2-C-C$\underline{H}$-N-) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 17.6, 18.4, 21.5, 25.8, 42.1, 46.8, 48.8, 63.2, 64.8, 65.1, 65.9, 70.2, 98.1, 123.2, 145.3, 172.5, 173.6 ppm. HRMS (ESI) m/z: [M+Na] + Calculated for $C_{83}H_{129}O_{34}N_9Na$ 1818.854; Found 1818.853

Synthesis of G2 **(24)**

To a mixture of G2 dendron **(20)** (1.437 g, 1.36 mmol) and tripropargyl-pentaerythritol **(6)** (0.095 g, 0.38 mmol) in THF (10 mL) was added sodium ascorbate (0.045 g, 0.226 mmol). After stirring for 5 minutes a solution of aqueous copper sulfate (0.028 g, 0.113 mmol in 1 mL of water) was added to the stirring mixture. The reaction was warmed at 40°C and stirred overnight under argon. Once the reaction was completed the organic solvent was removed *in vacuo*. The mixture was dissolved in DCM and extracted with water (x3), EDTA solution (x3) and brine (x1). The organic phase was dried with Na_2SO_4 and the product concentrated. The residue was purified by column chromatography (100% EtOAc to 5% MeOH in DCM) to give a sticky white foam **(24)** (1.113 g, 0.325 mmol, 86% yield). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 1.12 (s, -O-C-C$\underline{H}_3$, 36H), 1.24 (s, -CO-C-C$\underline{H}_3$, 18H), 1.33 (s, -CO-C-C$\underline{H}_3$, 36H), 1.39 (s, -O-C-C$\underline{H}_3$, 36H), 3.51 (s, N-C$\underline{H}_2$-CH_2-, 6H), 3.60 (d, -O-C$\underline{H}_2$-C-CO-, 24H), 4.12 (d, -O-C$\underline{H}_2$-C-CO-, HO-C$\underline{H}_2$-C-, 24H), 4.20-4.30 (m, -O-C$\underline{H}_2$-C-CO-, 36H), 4.55-4.66 (m, -N-CH_2-C$\underline{H}_2$-CO-,-O-C$\underline{H}_2$-C-CH-N-, -O-C$\underline{H}_2$-C- 18H), 7.72 (s, 3H, -O-CH_2-C-C$\underline{H}$-N-) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 14.2, 17.8, 18.4, 21.0, 25.2, 25.8, 42.1, 45.2, 46.8, 48.8, 53.4, 60.4, 63.2, 66.0, 70.2, 98.1, 123.0, 145.3, 171.6, 171.9, 173.6 ppm. HRMS (ESI) m/z: [M+Na]+ Calculated for $C_{161}H_{249}O_{70}N_9Na$ 3451.609; Found 3451.621

Synthesis of G1-pentyne **(25)**

G1 **(23)** (500 mg, 0.28 mmol), pentynoic acid (35 mg, 0.34 mmol) and DMAP (17 mg, 0.14 mmol) were dissolved in anhydrous DCM (10 mL), under argon atmosphere and

stirred for 10 minutes. Then DCC (288 mg, 1,40 mmol) and pyridine (5 mL) were added and the reaction was let to stir overnight at room temperature. The reaction mixture was diluted with DCM and extracted with water (x3), a saturated sodium carbonate solution (x3) and brine (x1). The organic phase was dried with sodium sulfate and filtered. The product was then concentrated and purified by column chromatography (100% EtOAc to 5% MeOH in DCM) to give a sticky white foam **(25)** (350 mg, 0.18 mol, 64% yield). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 1.23 (s, -O-C-C$\underline{H}_3$, 18H), 1.26 (s, -CO-C-C$\underline{H}_3$, 9H), 1.32 (s, -CO-C-C$\underline{H}_3$, 18H), 1.40 (s, -O-C-C$\underline{H}_3$, 18H), 2.09 (s, -O-CO-CH_2-CH_2-C-C$\underline{H}$, 1H), 2.50 (m, -O-CO-C$\underline{H}_2$-C$\underline{H}_2$-C-CH, 4H), 3.51 (t, N-C$\underline{H}_2$-CH_2-, 6H), 3.60 (d, -O-C$\underline{H}_2$-C-CO-, 12H), 4.12 (d, -O-C$\underline{H}_2$-C-CO-, HO-C$\underline{H}_2$-C-, 14H), 4.30 (dd, -O-C$\underline{H}_2$-C-CO-, 12H), 4.59 (m, -N-CH_2-C$\underline{H}_2$-CO-,-O-C$\underline{H}_2$-C-CH-N-, -O-C$\underline{H}_2$-C- 18H), 7.73 (s, 3H, -O-CH_2-C-C$\underline{H}$-N-) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 14.3, 17.6, 18.4, 21.5, 25.8, 33.1, 42.1, 46.8, 48.8, 63.2, 64.8, 65.1, 65.9, 70.2, 82.3, 98.1, 123.2, 145.3, 171.2, 172.5, 173.6 ppm. HRMS (ESI) m/z: [M+Na] + Calculated for $C_{88}H_{133}O_{35}N_9Na$ 1898.880; Found 1898.878

Synthesis of G2-pentyne **(26)**

G2 **(24)** (1.113 g, 0.33 mmol), pentynoic acid (40 mg, 0.40 mmol) and DMAP (25 mg, 0.20 mmol) were dissolved in anhydrous DCM (10 mL), under argon atmosphere and stirred for 10 minutes. Then DCC (82 mg, 0.40 mmol) and pyridine (2 mL) were added and the reaction was let to stir overnight at room temperature. The reaction mixture was diluted with DCM and extracted with water (x3), a saturated sodium carbonate solution

(x3) and brine (x1). The organic phase was dried with sodium sulfate and filtered. The product was then concentrated and purified by column chromatography (100% EtOAc to 5% MeOH in DCM) to give a sticky white foam **(26)** (700 mg, 0.20 mol, 61% yield). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 1.12 (s, -O-C-C$\underline{H}_3$, 36H), 1.24 (s, -CO-C-C$\underline{H}_3$, 18H), 1.33 (s, -CO-C-C$\underline{H}_3$, 36H), 1.39 (s, -O-C-C$\underline{H}_3$, 36H), 1.98 (s, -O-CO-CH_2-CH_2-C-C$\underline{H}$, 1H), 2.43-2.48 (m, -O-CO-C$\underline{H}_2$-C$\underline{H}_2$-C-CH, 4H), 3.50 (s, N-C$\underline{H}_2$-CH_2-, 6H), 3.61 (d, -O-C$\underline{H}_2$-C-CO-, 24H), 4.11 (d, -O-C$\underline{H}_2$-C-CO-, HO-C$\underline{H}_2$-C-, 24H), 4.20-4.30 (m, -O-C$\underline{H}_2$-C-CO-, 36H), 4.55-4.69 (m, -N-CH_2-C$\underline{H}_2$-CO-,-O-C$\underline{H}_2$-C-CH-N-, -O-C$\underline{H}_2$-C- 18H), 7.73 (s, 3H, -O-CH_2-C-C$\underline{H}$-N-) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 14.2, 17.8, 18.4, 21.0, 25.2, 25.8, 33.3, 42.1, 45.2, 46.8, 48.8, 53.4, 60.4, 63.2, 66.0, 70.2, 82.6, 98.1, 123.0, 145.3, 171.6, 171.9, 173.6 ppm. HRMS (ESI) m/z: [M+Na] + Calculated for $C_{166}H_{253}O_{71}N_9Na$ 3531.635; Found 3531.631

Synthesis of G1PEG2000 **(27)**

To a mixture of G1-pentyne **(25)** (144 mg, 0.077 mmol) and N_3PEG_{2000} **(11)** (154 mg, 0.070 mmol) in THF (5 mL) was added sodium ascorbate (4 mg, 0.015 mmol). After stirring for 5 minutes a solution of aqueous copper sulfate (2 mg, 0.008 mmol in 0.5 mL of water) was added to the mixture. The reaction was warmed at 40^{o}C overnight under argon atmosphere. Once the reaction was completed the THF was evaporated. The mixture was dissolved in DCM and solid ethylenediaminetetracetic acid disodium salt (Na_2EDTA) was added stirred vigorously for 2h. The solution was dried with Na_2SO_4 and stir for 30 min before filtration of the solids. The DCM was removed under reduced pressure and diethyl ether was added drop wise to precipitate the product. The ether was decanted and the

residue re-dissolved in DCM and the precipitation procedure repeated multiple times. The obtained product was a yellow sticky solid **(27)** (80 mg, 0.019 mmol, 28% yield). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 1.11 (s, -O-C-C$\underline{H}_3$, 18H), 1.25 (s, -CO-C-C$\underline{H}_3$, 9H), 1.33 (s, -CO-C-C$\underline{H}_3$, 18H), 1.41 (s, -O-C-C$\underline{H}_3$, 18H), 2.78 (d, -O-CO-C$\underline{H}_2$-CH_2-C-N, 2H), 3.03 (d, -O-CO-CH_2-C$\underline{H}_2$-C-N, 2H), 3.40 (s, -O-C$\underline{H}_3$, 3H), 3.51 (t, N-C$\underline{H}_2$-CH_2-, 6H), 3.60-3.75 (m, -O-C$\underline{H}_2$-C-CO-, -O-C$\underline{H}_2$-C$\underline{H}_2$-O-, 200H), 4.12 (d, -O-C$\underline{H}_2$-C-CO-, HO-C$\underline{H}_2$-C-, 14H), 4.28 (dd, -O-C$\underline{H}_2$-C-CO-, 12H), 4.55 (m, -N-CH_2-C$\underline{H}_2$-CO-,-O-C$\underline{H}_2$-C-CH-N-, -O-C$\underline{H}_2$-C-18H), 7.53 (s, 1H, -CH_2-CH_2-C-C$\underline{H}$-N-), 7.73 (s, 3H, -O-CH_2-C-C$\underline{H}$-N-) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 17.6, 18.5, 21.5, 25.6, 34.6, 42.1, 46.9, 48.9, 59.0, 63.1, 64.5, 65.2, 70.4, 71.7, 90.0, 98.1, 109.5, 122.4, 134.6, 163.1, 172.0, 173.6 ppm. GPC: Mn=4391 g/mol. Mw/Mn=1.12 MS (MALDI-TOF) m/z: [M+Na] + Calculated for $C_{191}H_{340}O_{86}N_{12}Na$ 4201.250; Found 4202.986

Synthesis of G2PEG2000 **(28)**

To a mixture of G2-pentyne **(26)** (350 mg, 0.10 mmol) and N_3PEG_{2000} **(11)** (200 mg, 0.09 mmol) in THF (5 mL) was added sodium ascorbate (5 mg, 0.02 mmol). After stirring for 5 minutes a solution of aqueous copper sulfate (3 mg, 0.01 mmol in 0.5 mL of water) was added to the mixture. The reaction was warmed at 40^{o}C overnight under argon atmosphere. Once the reaction was completed the THF was evaporated. The mixture was dissolved in DCM and solid ethylenediaminetetracetic acid disodium salt (Na_2EDTA) was added stirred vigorously for 2h. The solution was dried with Na_2SO_4 and stir for 30 min before filtration of the solids. The DCM was removed under reduced pressure and diethyl ether was added drop wise to precipitate the product. The ether was decanted and the

residue re-dissolved in DCM and the precipitation procedure repeated multiple times. The obtained product was a yellow sticky solid **(28)** (120 mg, 0.021 mmol, 23% yield). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 1.12 (s, -O-C-C$\underline{H}_3$, 36H), 1.22 (s, -CO-C-C$\underline{H}_3$, 18H), 1.33 (s, -CO-C-C$\underline{H}_3$, 36H), 1.40 (s, -O-C-C$\underline{H}_3$, 36H), 2.68 (t, -O-CO-C$\underline{H}_2$-CH_2-C-N, 2H), 2.95 (t, -O-CO-CH_2-C$\underline{H}_2$-C-N, 2H), 3.44 (s, -O-C$\underline{H}_3$, 3H), 3.48 (s, N-C$\underline{H}_2$-CH_2-, 6H), 3.55-3.75 (m, -O-C$\underline{H}_2$-C-CO-, -O-C$\underline{H}_2$-C$\underline{H}_2$-O-, 205H), 4.11 (d, -O-C$\underline{H}_2$-C-CO-, HO-C$\underline{H}_2$-C-, 24H), 4.20-4.30 (m, -O-C$\underline{H}_2$-C-CO-, 36H), 4.49-4.68 (m, -N-CH_2-C$\underline{H}_2$-CO-,-O-C$\underline{H}_2$-C-CH-N-, -O-C$\underline{H}_2$-C- 18H), 7.52 (s, 1H, -CH_2-CH_2-C-C$\underline{H}$-N-), 7.75 (s, 3H, -O-CH_2-C-C$\underline{H}$-N-) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 17.1, 17.5, 17.9, 18.5, 25.4, 30.9, 42.1, 46.9, 48.6, 48.8, 53.4, 59.0, 64.9, 70.4, 86.4, 98.1, 128.7, 171.9, 173.6 ppm. GPC: Mn=24773 g/mol. Mw/Mn=1.12 HRMS (MALDI-TOF) m/z: [M+H] + Calculated for $C_{225}H_{433}O_{115}N_{12}$ 5503.840; Found 5502.738

Synthesis of G2PEG6000 **(29)**

To a mixture of G2-pentyne **(26)** (350 mg, 0.10 mmol) and N_3PEG_{6000} **(12)** (539 mg, 0.07 mmol) in THF (3 mL) was added sodium ascorbate (4 mg, 0.02 mmol). After stirring for 5 minutes a solution of aqueous copper sulfate (3 mg, 0.01 mmol in 0.3 mL of water) was added to the mixture. The reaction was warmed at 40^{o}C overnight under argon atmosphere. Once the reaction was completed the THF was evaporated. The mixture was dissolved in DCM and solid ethylenediaminetetracetic acid disodium salt (Na_2EDTA) was added stirred vigorously for 2h. The solution was dried with Na_2SO_4 and stir for 30 min before filtration of the solids. The DCM was removed under reduced pressure and diethyl ether was added drop wise to precipitate the product. The ether was decanted and the

residue re-dissolved in DCM and the precipitation procedure repeated multiple times. The obtained product was a yellow solid **(29)** (724 mg, 0.067 mmol, 95% yield). ^{1}H-NMR (500MHz, $CDCl_3$): δ = 1.12 (s, -O-C-C$\underline{H}_3$, 36H), 1.22 (s, -CO-C-C$\underline{H}_3$, 18H), 1.33 (s, -CO-C-C$\underline{H}_3$, 36H), 1.40 (s, -O-C-C$\underline{H}_3$, 36H), 2.68 (t, -O-CO-C$\underline{H}_2$-CH_2-C-N, 2H), 2.95 (t, -O-CO-CH_2-C$\underline{H}_2$-C-N, 2H), 3.44 (s, -O-C$\underline{H}_3$, 3H), 3.48 (s, N-C$\underline{H}_2$-CH_2-, 6H), 3.55-3.75 (m, -O-C$\underline{H}_2$-C-CO-, -O-C$\underline{H}_2$-C$\underline{H}_2$-O-, 580H), 4.11 (d, -O-C$\underline{H}_2$-C-CO-, HO-C$\underline{H}_2$-C-, 24H), 4.20-4.30 (m, -O-C$\underline{H}_2$-C-CO-, 36H), 4.49-4.68 (m, -N-CH_2-C$\underline{H}_2$-CO-,-O-C$\underline{H}_2$-C-CH-N-, -O-C$\underline{H}_2$-C- 18H), 7.52 (s, 1H, -CH_2-CH_2-C-C$\underline{H}$-N-), 7.75 (s, 3H, -O-CH_2-C-C$\underline{H}$-N-) ppm. ^{13}C-NMR (125MHz, $CDCl_3$): δ = 15.3, 17.5, 17.9, 18.5, 20.7, 25.2, 25.8, 37.8, 42.1, 46.9, 48.6, 48.8, 53.4, 63.2, 64.9, 65.7, 68.9, 70.2, 70.4, 82.6, 98.1, 123.2, 145.3, 170.1, 171.9, 173.5 ppm. GPC: Mn=16291 g/mol. Mw/Mn=1.14 HRMS (MALDI-TOF) m/z: [M+H] + Calculated for $C_{481}H_{885}O_{228}N_{12}$ 10478.803; Found 10476.345

Attempted synthesis of G3 **(30)**

To a mixture of G3 dendron **(22)** (300 mg, 0.140 mmol) and tripropargyl-pentaerythritol **(6)** (10 mg, 0.04 mmol) in THF (5 mL) was added sodium ascorbate (6 mg, 0.03 mmol). After stirring for 5 minutes a solution of aqueous copper sulfate (4 mg, 0.015 mmol in 0.2 mL of water) was added to the stirring mixture. The reaction was warmed at 40ºC and stirred for 72 h under argon. A sticky brown precipitate was forming in the round bottom flask. The reaction was stopped and the THF evaporated. The precipitate was triturate with water, EDTA solution and EtOAc. The product obtained was a brown solid.

2.5 - References

1. Wurm, F.; Frey, H., Linear–dendritic block copolymers: The state of the art and exciting perspectives. *Prog. Polym. Sci.* **2011,** *36* (1), 1-52.
2. Bucknall, D. G.; Anderson, H. L., Polymers Get Organized. *Science* **2003,** *302* (5652), 1904-1905.
3. Liu, X. Y.; Nothias, J.-M.; Scavone, A.; Garfinkel, M.; Millis, J. M., Biocompatibility Investigation of Polyethylene Glycol and Alginate-Poly-l-Lysine for Islet Encapsulation. *ASAIO Journal* **2010,** *56* (3), 241-245.
4. Feliu, N.; Walter, M. V.; Montañez, M. I.; Kunzmann, A.; Hult, A.; Nyström, A.; Malkoch, M.; Fadeel, B., Stability and biocompatibility of a library of polyester dendrimers in comparison to polyamidoamine dendrimers. *Biomaterials* **2012,** *33* (7), 1970-1981.
5. Blasco, E.; Piñol, M.; Oriol, L., Responsive Linear-Dendritic Block Copolymers. *Macromol. Rapid Commun.* **2014,** *35* (12), 1090-1115.
6. Pasut, G.; Veronese, F. M., PEG conjugates in clinical development or use as anticancer agents: An overview. *Advanced Drug Delivery Reviews* **2009,** *61* (13), 1177-1188.
7. Pasut, G.; Veronese, F. M., Polymer–drug conjugation, recent achievements and general strategies. *Prog. Polym. Sci.* **2007,** *32* (8–9), 933-961.
8. Mero, A.; Clementi, C.; Veronese, F. M.; Pasut, G., Covalent conjugation of poly(ethylene glycol) to proteins and peptides: strategies and methods. *Methods in molecular biology (Clifton, N.J.)* **2011,** *751*, 95-129.
9. Parveen, S.; Sahoo, S., Nanomedicine. *Clin Pharmacokinet* **2006,** *45* (10), 965-988; Blume, G.; Cevc, G., Liposomes for the sustained drug release in vivo. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1990,** *1029* (1), 91-97.
10. Kwon, G. S.; Kataoka, K., Block copolymer micelles as long-circulating drug vehicles. *Advanced Drug Delivery Reviews* **1995,** *16* (2–3), 295-309.
11. Sousa-Herves, A.; Riguera, R.; Fernandez-Megia, E., PEG-dendritic block copolymers for biomedical applications. *New J. Chem.* **2012,** *36* (2), 205-210.
12. Carlmark, A.; Malmstrom, E.; Malkoch, M., Dendritic architectures based on bis-MPA: functional polymeric scaffolds for application-driven research. *Chem. Soc. Rev.* **2013,** *42* (13), 5858-5879.
13. Albertazzi, L.; Gherardini, L.; Brondi, M.; Sulis Sato, S.; Bifone, A.; Pizzorusso, T.; Ratto, G. M.; Bardi, G., In Vivo Distribution and Toxicity of PAMAM Dendrimers in the Central Nervous System Depend on Their Surface Chemistry. *Molecular Pharmaceutics* **2013,** *10* (1), 249-260.
14. Johansson, M.; Malmström, E.; Hult, A., Synthesis, characterization, and curing of hyperbranched allyl ether–maleate functional ester resins. *J. Polym. Sci. A Polym. Chem.* **1993,** *31* (3), 619-624.
15. Ihre, H.; Hult, A.; Söderlind, E., Synthesis, Characterization, and 1H NMR Self-Diffusion Studies of Dendritic Aliphatic Polyesters Based on 2,2-Bis(hydroxymethyl)propionic Acid and 1,1,1-Tris(hydroxyphenyl)ethane. *J. Am. Chem. Soc.* **1996,** *118* (27), 6388-6395.
16. Ihre, H.; Hult, A.; Fréchet, J. M. J.; Gitsov, I., Double-Stage Convergent Approach for the Synthesis of Functionalized Dendritic Aliphatic Polyesters Based on 2,2-Bis(hydroxymethyl)propionic Acid. *Macromolecules* **1998,** *31* (13), 4061-4068.

17. Malkoch, M.; Malmström, E.; Hult, A., Rapid and Efficient Synthesis of Aliphatic Ester Dendrons and Dendrimers. *Macromolecules* **2002,** *35* (22), 8307-8314.
18. Ihre, H.; Padilla De Jesús, O. L.; Fréchet, J. M. J., Fast and Convenient Divergent Synthesis of Aliphatic Ester Dendrimers by Anhydride Coupling. *J. Am. Chem. Soc.* **2001,** *123* (25), 5908-5917.
19. García-Gallego, S.; Hult, D.; Olsson, J. V.; Malkoch, M., Fluoride-Promoted Esterification with Imidazolide-Activated Compounds: A Modular and Sustainable Approach to Dendrimers. *Angew. Chem. Int. Ed.* **2015,** *54* (8), 2416-2419.
20. Kolb, H. C.; Finn, M. G.; Sharpless, K. B., Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew. Chem. Int. Ed.* **2001,** *40* (11), 2004-2021.
21. Rolf Huigsen, Centenary Lecture 1,3-Dipolar Cycloadditions. October 1961. *Proc. Chem. Soc.* **1961,** (October), 357-396.
22. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B., A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes. *Angew. Chem. Int. Ed.* **2002,** *41* (14), 2596-2599.
23. Tornøe, C. W.; Christensen, C.; Meldal, M., Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. *J. Org. Chem.* **2002,** *67* (9), 3057-3064.
24. 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., Efficiency and Fidelity in a Click-Chemistry Route to Triazole Dendrimers by the Copper(I)-Catalyzed Ligation of Azides and Alkynes. *Angew. Chem. Int. Ed.* **2004,** *43* (30), 3928-3932.
25. Sharma, A.; Neibert, K.; Sharma, R.; Hourani, R.; Maysinger, D.; Kakkar, A., Facile Construction of Multifunctional Nanocarriers Using Sequential Click Chemistry for Applications in Biology. *Macromolecules* **2011,** *44* (3), 521-529; Franc, G.; Kakkar, A., Dendrimer design using CuI-catalyzed alkyne-azide "click-chemistry". *Chem. Commun.* **2008,** (42), 5267-5276.
26. Lee, J.; Han, S.; Kim, B.-K.; Lee, U.; Sung, S.; Kang, H.-S.; Kim, J.; Jin, S.-H., Facile synthesis of dendritic-linear-dendritic materials by click chemistry. *Macromol. Res.* **2009,** *17* (7), 499-505.
27. Chatani, S.; Podgórski, M.; Wang, C.; Bowman, C. N., Facile and Efficient Synthesis of Dendrimers and One-Pot Preparation of Dendritic–Linear Polymer Conjugates via a Single Chemistry: Utilization of Kinetically Selective Thiol–Michael Addition Reactions. *Macromolecules* **2014,** *47* (15), 4894-4900.
28. Javakhishvili, I.; Binder, W. H.; Tanner, S.; Hvilsted, S., Facile synthesis of linear-dendritic cholesteryl-poly([varepsilon]-caprolactone)-b-(l-lysine)G2 by thiol-ene and azide-alkyne "click" reactions. *Polym. Chem.* **2010,** *1* (4), 506-513.
29. Kempe, K.; Onbulak, S.; Schubert, U. S.; Sanyal, A.; Hoogenboom, R., pH degradable dendron-functionalized poly(2-ethyl-2-oxazoline) prepared by a cascade "double-click" reaction. *Polym. Chem.* **2013,** *4* (11), 3236-3244.
30. Lutz, J.-F., Copper-Free Azide–Alkyne Cycloadditions: New Insights and Perspectives. *Angew. Chem. Int. Ed.* **2008,** *47* (12), 2182-2184.
31. Agard, N. J.; Baskin, J. M.; Prescher, J. A.; Lo, A.; Bertozzi, C. R., A Comparative Study of Bioorthogonal Reactions with Azides. *ACS Chemical Biology* **2006,** *1* (10), 644-648.

32. Ornelas, C.; Broichhagen, J.; Weck, M., Strain-Promoted Alkyne Azide Cycloaddition for the Functionalization of Poly(amide)-Based Dendrons and Dendrimers. *J. Am. Chem. Soc.* **2010,** *132* (11), 3923-3931.
33. Li, Y.; Giles, M. D.; Liu, S.; Laurent, B. A.; Hoskins, J. N.; Cortez, M. A.; Sreerama, S. G.; Gibb, B. C.; Grayson, S. M., A versatile and modular approach to functionalisation of deep-cavity cavitandsvia "click" chemistry. *Chem. Commun.* **2011,** *47* (32), 9036-9038.
34. Neises, B.; Steglich, W., Simple Method for the Esterification of Carboxylic Acids. *Angew. Chem. Int. Ed. Eng.* **1978,** *17* (7), 522-524.
35. Surh, I.; Behl, M.; Elmore, S. A.; Chhabra, R. S., Comparative dermal toxicity of dicyclohexylcarbodiimide and diisopropylcarbodiimide in rodents. *Cutaneous and ocular toxicology* **2012,** *31* (3), 177-87.
36. Curtis, W. The design and synthesis of dendrons and dendronized polymers for applications in the pulp and paper industry. McGill University, 2014.
37. Lu, X.; Bittman, R., Synthesis of a Photoactivatable (2S,3R)-Sphingosylphosphorylcholine Analogue. *J. Org. Chem.* **2005,** *70* (12), 4746-4750.

Chapter 3: Self-Assembly, pH Responsiveness and Drug Delivery using Linear Dendritic Block Copolymers

3.1 - Introduction

The self-assembly of amphiphilic block copolymers has been extensively investigated, and constitutes an interesting area for applications in a diverse range of topics including catalysis,[1] drug delivery.[2] etc. Due to a wide variety of polymers available, polymeric micelles can be easily tailored for the intended application.[3] Amphiphilic linear dendritic block copolymers (LDBC) are composed of a linear polymer and a dendron, which offer a new platform to fine tune the introduction of desired entities in the formed supramolecular structures (Figure 3.1). The multiple surface groups of a dendron are suitable for functionalization and thus increase the potential applications of self-assembled structures.[4] For drug delivery applications, LDBC allow controlled incorporation of drug or stimuli sensitive group at the surface the dendron, in comparison to linear polymers which rely on statistical distribution.[5] Furthermore, self-assembled LDBC show lower critical micelle concentration (CMC), which has been associated with longer circulating time and better colloidal stability, and increase drug loading, compared to the linear block copolymers, which make them especially valuable for biological applications.[6] The lower CMC of linear dendritic block copolymers has been explained, through Brownian dynamic simulations, by Cao and co-workers as being inversely proportional to the branching parameter of the hydrophobic core.[7] In theory, the more branched the dendron of the LDBC

is, the lower its CMC. These improved properties make self-assembly of LDBCs an interesting area of research with a lot of potential.

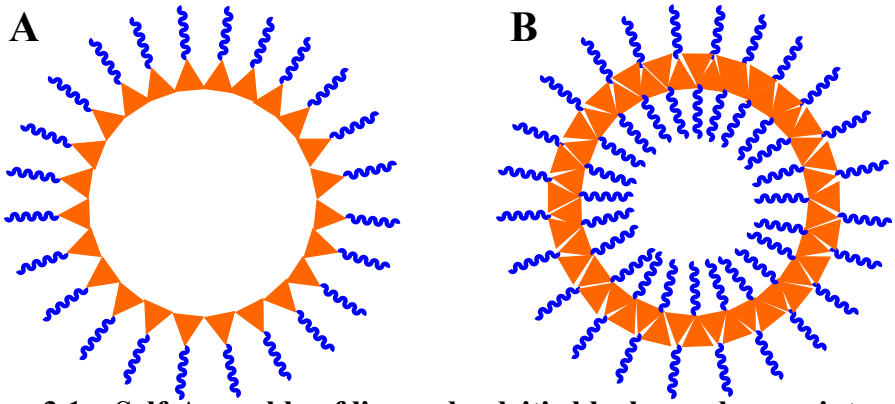


Figure 3.1 – Self-Assembly of linear dendritic block copolymers into a micelle (A) and a vesicle (B).

The first LDBC reported by Fréchet and Gitsov in 1992, was composed of polybenzylether dendron and PEG, and it was self-assembled in methanol and studied by ^{1}H-NMR spectroscopy.[8] From the molecular dynamics of the PEG chain, the broadness of the signal was interpreted as an evidence of limited flexibility, it was deducted that micelles were formed from this LDBC. Now, DLS and TEM are standard tools to study self-assembled micelles.[9] In general spherical micelles between 20 and 100 nm with high polydispersity index (0.2-0.4) are obtained from LDBC self-assembly,[10-12] however two examples stand out from the literature for their unexpected nanostructures. In 1995, Meijer studied self-assembly of LDBC based on polystyrene chain and poly(propylene imine) dendron.[13] The dendritic generation was increased while keeping the polystyrene chain of the same length. The results gave a very strong generation dependant self-assembly with nanostructure showing vesicles (G2), rod-like micelle (G3) and spherical micelles (G4). More recently del Barrio et al.., reported the self-assembly of LDBC made of PEG and bis-MPA dendron functionalized with cyanoazobenzene.[14] These self-assembled LDBC gave

polymersomes (or dendrimersomes in this case), sheet like structure, and tubular micelles (Figure 3.2). This wide range of structures followed Israelachvili theory, and were affected by the ratio of hydrophobic to hydrophilic, with an increasing hydrophobic fraction yielding structures ranging from tubular micelles to sheet like micelles to polymeric vesicles.[15]

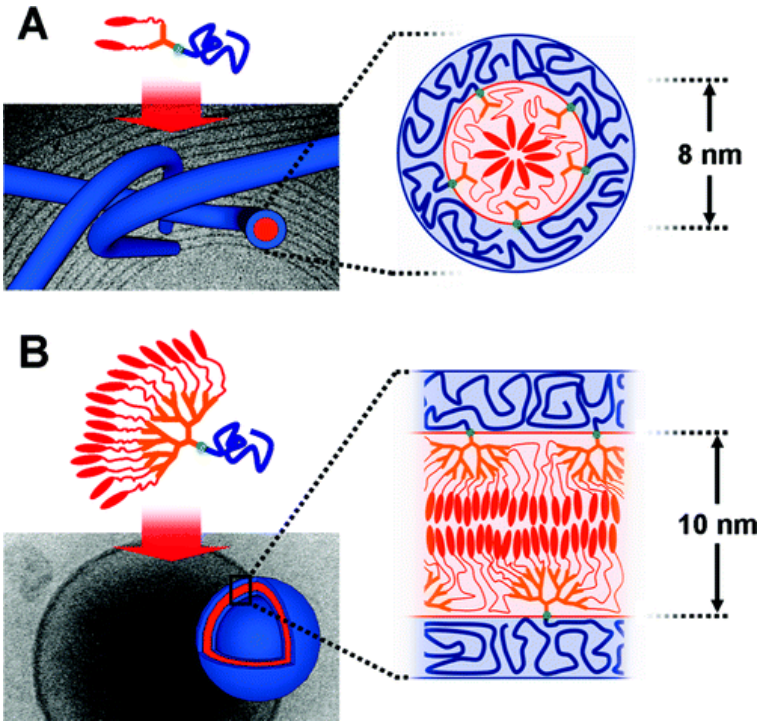


Figure 3.2 – Schematic representation of the chain structures and the linear dendritic block copolymer self-assemblies. Reprinted with permission from reference [14]. Copyright 2010 American Chemical Society.

Self-assembled materials that respond to certain stimuli are of great interest,[16] particularly for drug delivery systems as they allow to control delivery of drug both spatially and temporally.[17] The most commonly explored stimuli are light[18], heat[19] and pH[20]. Light responsive LDBC are usually prepared by decorating the surface of the dendron with a light sensitive group.[21] Azo-benzene is commonly used for this purpose,[22] but Dong

and co-workers reported the use of diazonaphtoquilone (DNQ) on the surface. DNQ is a hydrophobic moiety which undergoes light induced Wolff rearrangement[23] under UV or NIR irradiation and becomes hydrophilic. This drastic change in polarity has been used to its advantage to change the polarity on the surface of the PAMAM dendron (Figure 3.3). When the micellar solution was irradiated, micelles were disrupted due to LDBC dissolving in water and thus released its content, in this case doxorubicin. The *in vitro* studies showed an NIR-triggered cytotoxicity proving the efficiency of this type of stimuli responsive drug delivery system.[24]

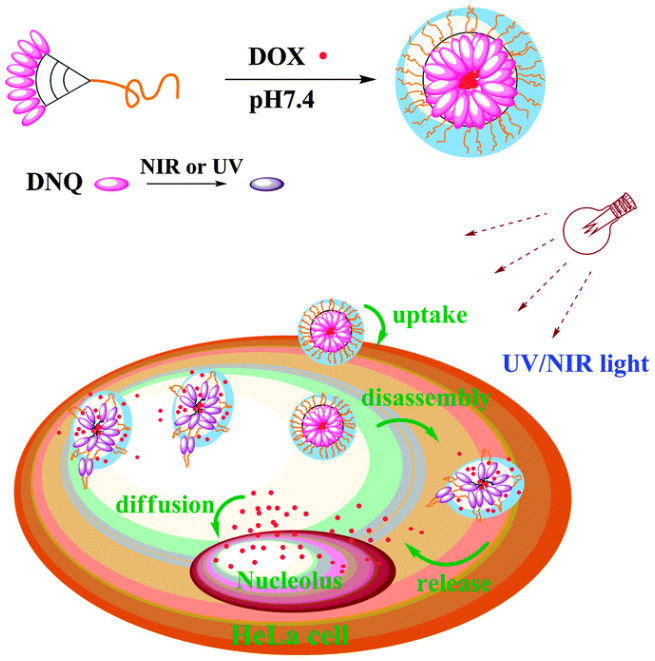


Figure 3.3 – DNQ functionalized LDBC Self-Assembly and their use as drug delivery system. Reprinted with permission from reference [24]. Copyright 2014 Royal Society of Chemistry.

In thermoresponsive LDBCs the linear part can be functionalized to respond to temperature change. Liu et al. reported the synthesis of a LDBC containing poly(N-

isopropylacrylamide) (PNIPAM), a polymer which solubility in water drops above 32ºC, and poly(benzylether) dendron.[25] It was then self-assembled into micelles at 20ºC and the micellar solution was heated, resulting in micelles collapsing and aggregating.

pH responsive LDBCs have been reported by Fréchet and co-workers using PEG and poly(L-lysine) dendron bearing a pH sensitive trimethoxybenzaldehyde acetal groups at its surface.[11] LDBCs were self-assembled into micelles and diluted in a pH 5 buffer. The effect was monitored only by DLS and showed a decrease in the size followed by disappearance of the micelles, after a few hours. The kinetic study showed that the contents of the micelle were released faster at acidic pH.

The potential of such stimuli responsive systems in drug delivery is tremendous.[26] Chen and co-workers reported the self-assembly of pH responsive PEG-PAMAM LDBC and its *in vitro* effect as a drug delivery system.[27] The surface of the dendron was functionalized with doxorubicin via pH sensitive hydrazone bond, while the micelle was loaded with another anticancer drug, camptothecin. The drug loading inside the micelle was high due to both conjugation and encapsulation, and the combination effect of these two drugs showed increase in apoptosis of cancer cells compared to unloaded micelles.

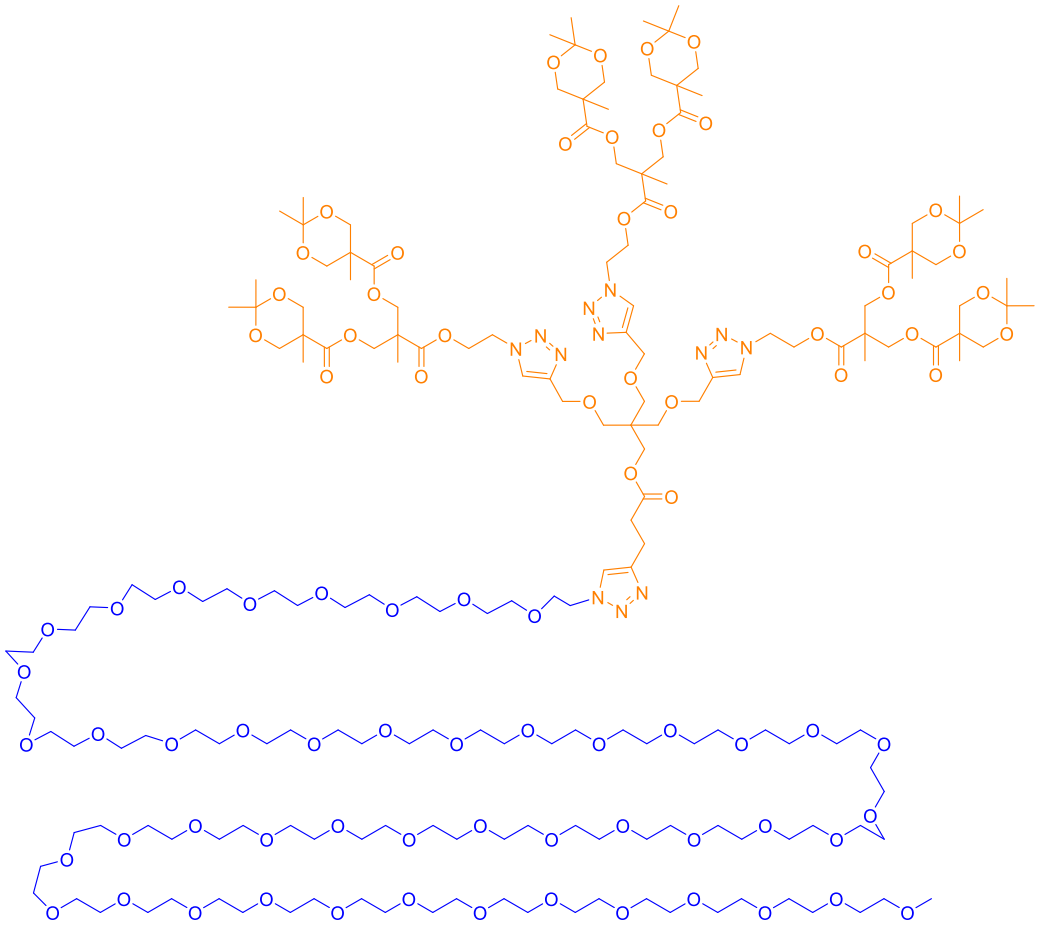
We have examined the self-assembly behavior of LDBC synthesized in Chapter 2. Micellar structures are the most reported type of self-assembly for LDBC, and we expected the same type of behavior in our systems.[10] The pH responsiveness of the micellar LDBC was monitored by TEM, which is a very useful technique to visualize this behavior. Finally, the potential of the self-assembled LDBC for drug delivery was explored by encapsulating acetazolamide (ATZ), which is a carbonic anhydrase inhibitor with potential in cancer

therapy,[28] and treating tumor cell spheroids with the loaded micelles. The *in vitro* results show the great potential of the self-assembled LDBC as a drug delivery system.

3.2 - Results and discussion

3.2.1 - Structural characteristic

The LDBC employed in this study are amphiphilic in nature as shown in Scheme 3.1, representing G1PEG2000 with the fully extended PEG chain. The asymmetric aspect of this amphiphilic macromolecule can be clearly visualized. The hydrophobic part is short and condensed. On the other hand, the hydrophilic part is long and linear.



Scheme 3.1 - Extended structure of G1PEG2000.

For the linear diblock copolymers, the hydrophobic to hydrophilic fraction ratio can be used to predict the type of self-assembled structure that will be obtained, using the Israelachvili theory.[15] For example in linear block copolymers, micelles are obtained with a hydrophobic/hydrophilic ratio of 15/85 to 30/70. The self-assembly is affected by other factors including the packing parameters, but the ratio gives a general idea of the possible outcome.[29] On the other hand, in LDBC self-assembly, the asymmetric structure and the compact dendritic part, in comparison to symmetric long linear chains, affect the outcome. Table 3.1 recapitulates all the structural information of the prepared LDBCs and shows the wide range of hydrophobic/hydrophilic ratios explored.

Table 3.1 - MW distribution in LDBC

LDBC	Total MW	Hydrophobic MW	Hydrophilic MW	Hydrophobic / Hydrophilic
G1PEG2000	4077	1877	2200	46/54
G2PEG2000	5730	3510	2200	60/40
G2PEG6000	10010	3510	6500	36/64

3.2.2 - Self-Assembly

The self-assembly studies were performed using co-solvent evaporation method, which is widely used to self-assemble amphiphilic block copolymers.[30] First the LDBCs (0.15 – 1.5 mg) were dissolved in acetone (1.0 mL) a good solvent for both the hydrophobic and hydrophilic part. The solution was then added dropwise to stirring deionized water (1.5 mL), a selective solvent for the hydrophilic chain. Acetone was left to evaporate overnight and samples were then analysed by dynamic light scattering (DLS) to determine the hydrodynamic diameter (Rh) of the formed nanoparticles (Table 3.2). It is important to

note that the self-assembly behavior of G1PEG2000 and G2PEG6000 LDBCs was studied at 1.0 mg/mL. However, at this concentration, G2PEG2000 formed a cloudy solution and the concentration was subsequently reduced to 0.1 mg/mL for this LDBC. When a similar concentration was used for G1PEG2000 and G2PEG6000, no significant difference between 0.1 and 1.0 mg/mL was observed in the DLS results.

Table 3.2 - DLS data for self-assembled LDBC

LDBC	Hydrodynamic Radius (Rh) ± SD	Polydispersity Index (PDI) ± SD
G1PEG2000	173.51 ± 0.24	0.091 ± 0.009
G2PEG2000	163.12 ± 1.05	0.049 ± 0.006
G2PEG6000	322.87 ± 2.44	0.177 ± 0.008

DLS analysis showed the presence of monodisperse nanoparticles, with PDI below 0.1, for G1PEG2000 and G2PEG2000 and large hydrodynamic diameter in comparison to other self-assembled LDBC.[11] The size as well as the polydispersity for G2PEG6000 were higher, and this behavior could be explained using the transmission electron microscopic (TEM) images. LDBCs are organic molecules and TEM imaging of this type of systems can be problematic since the electron density in these materials is low. One way to increase the electron density is by staining the organic sample with heavier atoms. The most commonly used staining agent is uranyl acetate (UAc_2)[31] and we used it to stain the self-assembled LDBCs, however the results obtained showed a tubular network (Figure 3.4). This tubular network was in direct contradiction with the DLS data, which indicates discrete monodisperse nanostructures.

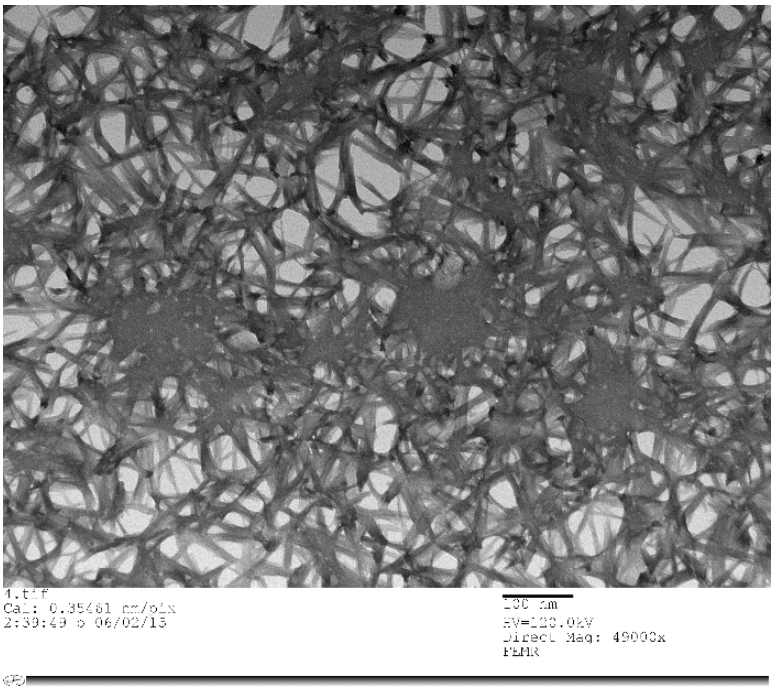


Figure 3.4 - TEM image of G1PEG2000 with UAc_2 stain.

The problem originated from the pH of the UAc_2 solution used. In fact, the staining solution pH was about 4.5, and at this pH the acetonide groups are deprotected which lead to the formation of a crystalline structure. Since uranyl acetate precipitates when its pH is neutralized, a less acidic staining solution was prepared based on phosphotungstic acid ($H_3PW_{12}O_{40}$). Its pH was carefully adjusted to 6.5 using sodium hydroxide solution. If the pH is higher than 7, the staining agent starts degrading. With the suitable staining agent, good TEM images of the self-assembled LDBCs were obtained (Figure 3.5 & 3.6).

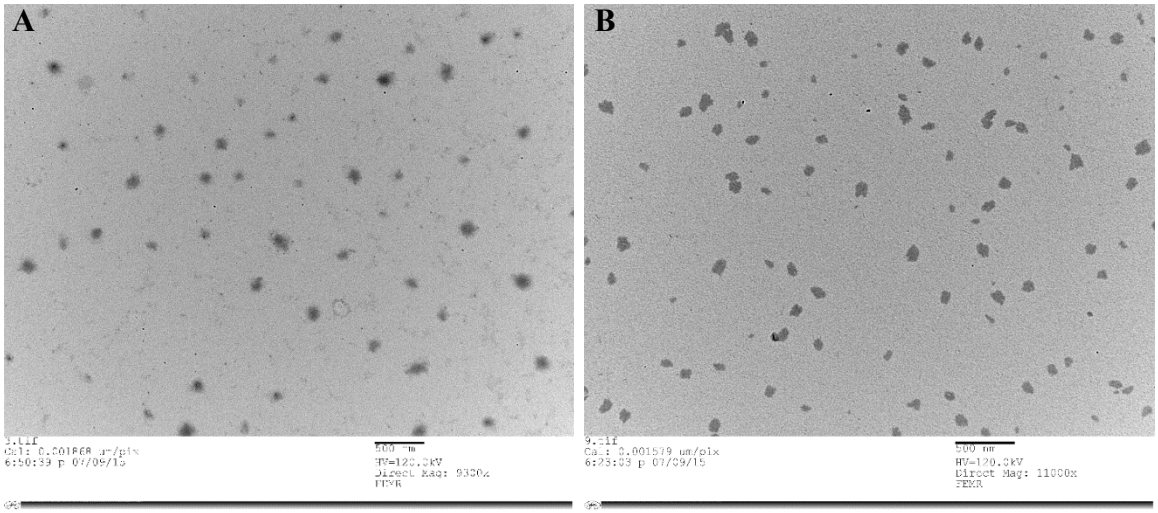


Figure 3.5 - TEM images of G1PEG2000 micelles (A) and G2PEG2000 micelles (B).

As we can see from the TEM images, micelles are formed for both G1PEG2000 and G2PEG2000, the sizes of which were about 100 nm, which are consistent with the DLS data. The lower micellar size in TEM, in comparison to the DLS, a solution measurement, maybe due to shrinking of the sample during drying on the TEM grid. It should be noted that the micelles were monodisperse and non-aggregated.

For G2PEG6000 a different type of self-assembled structure was obtained, and although the rod-like structure was unexpected, it is in agreement with the DLS data. The high polydispersity index observed was due to the rode-like shape of the self-assembled system. DLS measured the diameter for both the width and the length of the rod like micelles, increasing the PDI. The longer PEG chain of G2PEG6000 compared to G2PEG2000 seems to affects the self-assembly dramatically.

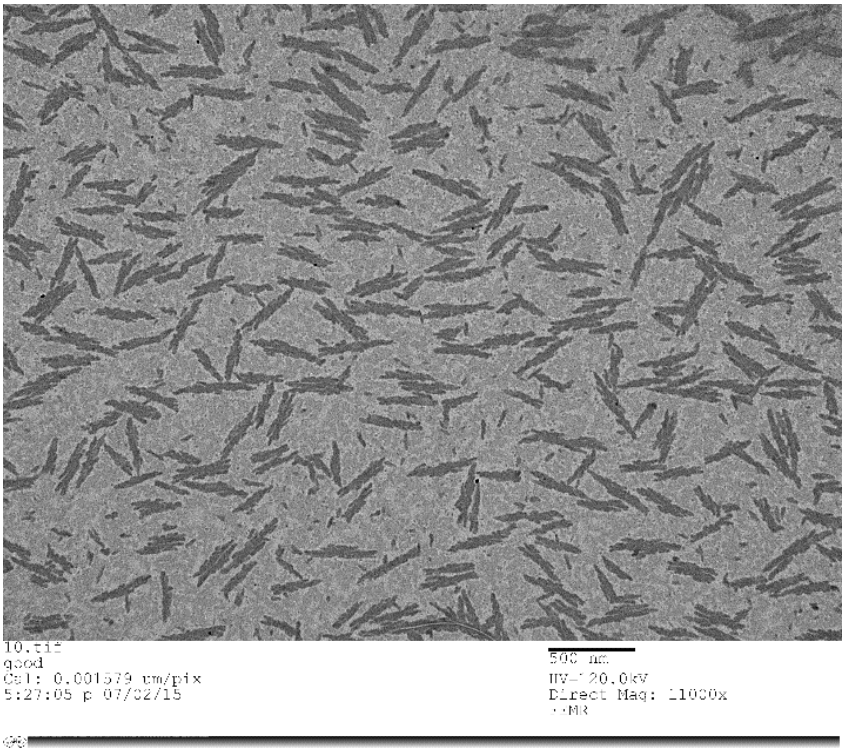


Figure 3.6 – TEM image of rod-like micelles from G2PEG6000.

3.2.3 - pH study and CMC determination

Since, it has been shown that smaller micelles exhibit better delivery efficacy,[32] G1PEG2000, which had the smallest particle size, was further studied as a drug delivery system. However, before the biological studies the pH responsiveness of the self-assembled G1PEG2000 micelles was tested to ensure the efficiency of the drug delivery system. The acetonide groups on the surface of the dendron make the surface hydrophobic and are pH sensitive. At pH 5, they should start to deprotect and expose the diols. This change on the surface of the dendron is expected to affect the self-assembled micelle. The pH effect was studied using two different methods: DLS and TEM. Aliquots of micellar solutions were diluted in pH 5 and pH 4 buffer solution and the DLS recorded over time (Figure 3.7).

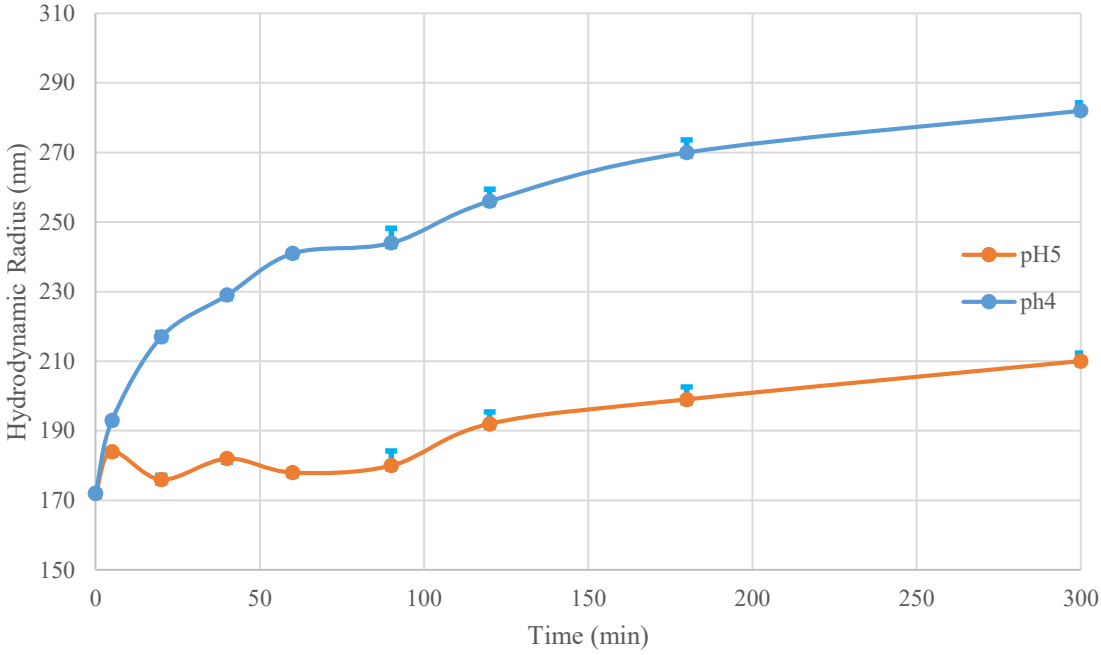


Figure 3.7 - Hydrodynamic radius of G1PEG2000 micelles in mild acidic solution.

As we can see in Figure 3.7, when subjected to acidic solution the hydrodynamic radius of the micelle started to increase. The polydispersity also increased from 0.1 to 0.14 over 300 minutes in both cases. Since polydispersity did not increase as drastically as the hydrodynamic diameter, it is proposed that the micelles swell as the acetonide group are deprotected. The increase in diameter of the micelle was not very high at pH 5 but was still significant, while at pH 4 the micellar size increased by 100 nm over a period of just 3 hours. Finally, the DLS measurement after 24h and one week, at these pH, were also recorded and the results are summarized in Table 3.3. We can see that after 24h the difference between pH 4 and 5 was not large, likely due to the deprotection of all acetonide groups. After 1 week, large aggregates were found at both pH.

Table 3.3 - pH effect on the hydrodynamic radius (Rh) and polydispersity (PDI)				
pH	t=0	t=5h	t=24h	t=1 week
4	Rh=172 ± 0.75 PDI=0.102 ± 0.006	Rh=282 ± 2.3 PDI=0.163 ±	Rh=352 ± 2.2 PDI=0.16 ± 0.039	Rh=642 ± 48 PDI=0.243 ± 0.044
5	Rh=172 ± 0.75 PDI=0.102 ± 0.006	Rh=210 ± 0.79 PDI=0.028 ± 0.017	Rh=345 ± 0.214 PDI=0.195 ± 0.034	Rh=479 ± 16.19 PDI=0.243 ± 0.024

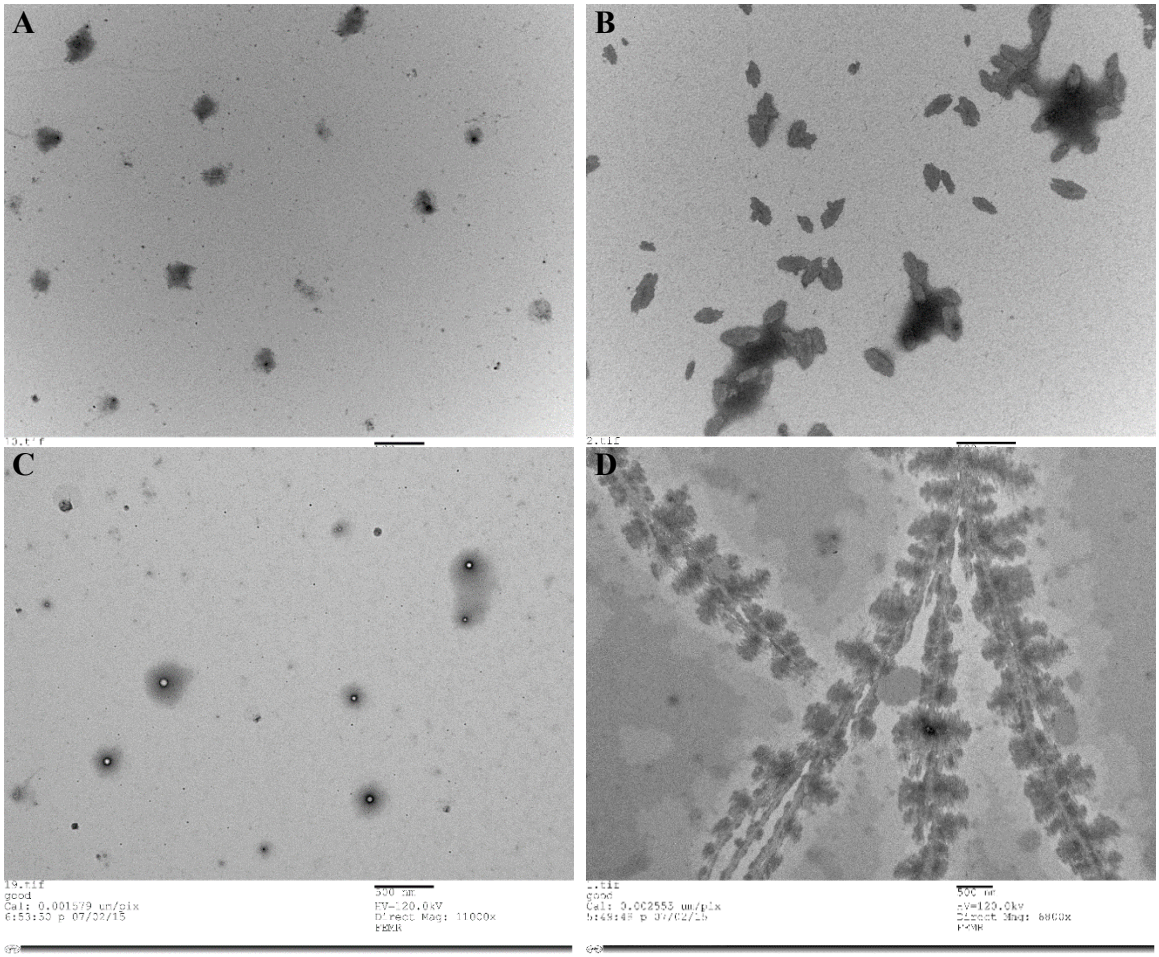
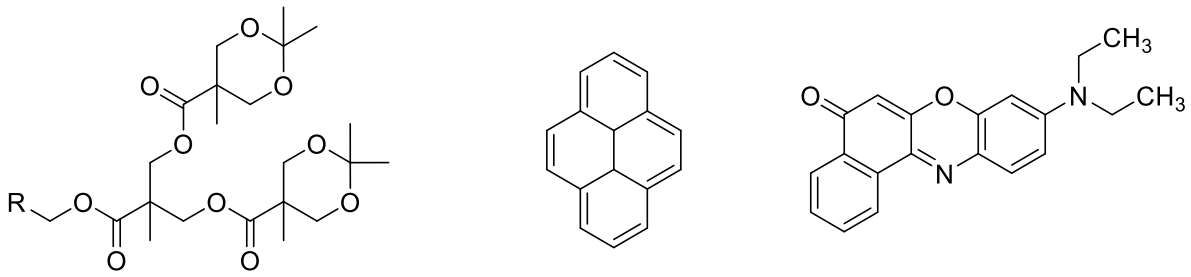


Figure 3.8 – TEM image of G1PEG2000 micelles at pH 4 for 1h (A), 2h (B), 4h (C), 24h (D).

The TEM study suggested swelling of the micelles at acidic pH (Figure 3.8). After 1 hour treatment, micelles were approximately 150-200 nm, which is double the size of the starting micelles. After 2 hours, even bigger micelles can be seen and some of them start to aggregate. It should also be noted that micelles experienced a significant change in shape, shifting from circular to ellipsoidal nanostructure (Figure 3.8 B). The 4 hours image (Figure 3.8 C) shows that larger aggregates are formed, with a diameter of about 220-250 nm which is in good agreement with the DLS data. Finally, the last figure, after 24 hours exposure to acidic pH, shows a crystalline structure. It seems to be composed of a thick main fiber on which crystals are growing. This crystalline structure suggests that the deprotection of the diols leads to higher ordering of the nanostructure.

We subsequently attempted to determine the critical micellar concentration (CMC), also known as critical aggregation concentration (CAC) of self-assembled G1PEG2000. The CMC in LDBC micelles is usually lower than linear block copolymer micelles[33], which is an advantage for drug delivery applications, since low CMC is synonym for better stability with the system and thus longer circulating time[34]. The CMC determination is commonly done by introducing a pyrene molecule inside micelles and measuring the fluorescence.[33] Pyrene is a fluorescent molecule which has different emission and excitation spectra depending on its environment. When switching from a polar environment, such as water, to a less polar one, for example the interior of a micelle, a significant shift occurs in its spectra. To introduce the molecular probe inside the micelle, aliquots of different micelle concentration (from 0.01 mg/L to 200 mg/L) were stirred overnight in a solution of pyrene in water (16 μM). The excitation spectra was then measured for an emission at 390 nm, and the ratio of intensity at 331 to 334 nm was

calculated.[35] Unfortunately, no change were observed in the spectra over the whole range of micellar concentration. To ensure the presence of micelles in the tested solutions, DLS experiments were performed. DLS was able to detect micelles for 200 µg/mL although there was no change in the fluorescence spectra which ruled out the possibility of a high CMC. Two other options might explain the lack of change in the emission spectra. First, the pyrene is unable to access the micelle core once they are self-assembled which can be explained by highly packed micelles. Second possibility, is that the micellar core environment is not favorable for pyrene to be introduced inside. The answer is probably a bit of both. The entropy of pyrene inside the micelle is likely lower than in aqueous solution due to packing and the possible enthalpy gain for interacting with a more hydrophobic environment does not overcome the entropic loss of entering the micelle. This could be explained by comparing the pyrene structure to the surface group (scheme 3.2), the pyrene is a fully aromatic molecule while the surface of the dendron (the micellar core) is made of esters and aliphatic groups. The difference between the two systems might explain that pyrene does not enter the micelles.



Scheme 3.2 - Comparison of dendritic molecular structure to pyrene and nile red.

Fortunately, other molecules can be used to determine the CMC. Nile red[11], benzoylacetone[36] and 1-(2-pyridylazo)-2-naphtol[37] are some of them. These molecules

were not tested due to lack of time, but are likely to work better as they are more polar than pyrene and should have better interaction with the micelle core.

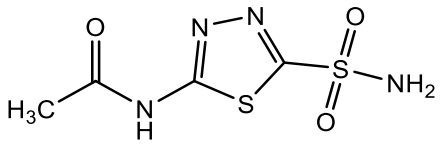
3.2.4 - Biological Study

The potential of G1PEG2000 micelles as a drug delivery system was subsequently investigated. Acetazolamide (ATZ) (Scheme 3.3), a carbonic anhydrase inhibitor with potential in cancer treatment, was encapsulated inside the micelle and its efficacy tested *in vitro*. The encapsulation of ATZ was carried out by diluting the drug with acetone solution of LDBC and following the same procedure as for blank micelle preparation. Various amounts of ATZ were diluted in an acetone solution, and after self-assembly the solution was filtered to remove unencapsulated drug. DLS studies showed no significant difference in the size and PDI of the micelle. Aliquots of loaded micellar solution were diluted in methanol and HPLC was run to determine the amount of drug in each sample. The concentration was then plotted against standard solution of drug, measured prior to running samples. The encapsulation efficiency and the loading capacity of the micelle were calculated from the HPLC data and equation 1 and 2. The data are summarized in table 3.4 and show the high loading capacity of our system.

$$Encapsulation\ efficiency\ \% = \frac{Weight\ of\ ATZ\ in\ micelle}{Initial\ weight\ of\ ATZ} \quad (1)$$

$$Loading\ capacity\ \% = \frac{Weight\ of\ ATZ\ in\ micelle}{Total\ weight\ of\ micelles} \quad (2)$$

Table 3.4 - Drug loading and encapsulation		
Drug/Polymer Ratio	Encapsulation Efficiency	Loading Capacity
30%	72%	25%
50%	71%	35%
100%	88%	57%



Acetalozamide (ATZ)

Scheme 3.3 - Acetazolamide molecular structure.

Biological studies were performed using U251N tumor cell spheroids which were treated with ATZ loaded micelle, and the results were compared with ATZ alone treatment at the same concentration, over a period of one day.

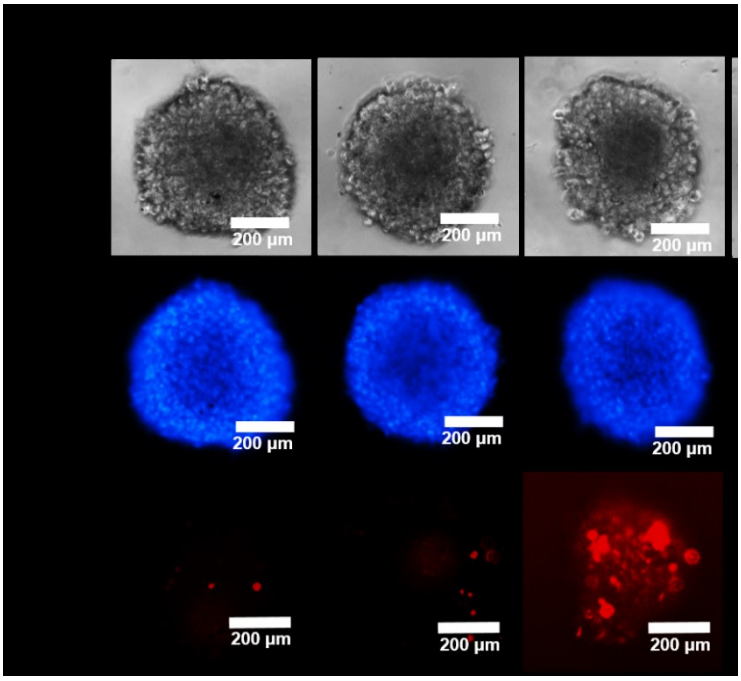


Figure 3.9 – Spheroids imaging after one day treatment.

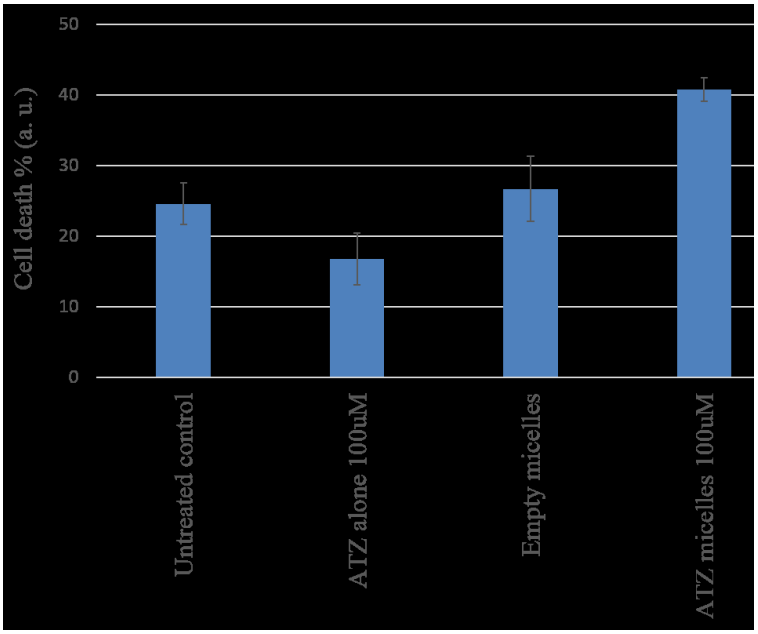


Figure 3.10 – Cell death after one day treatment.

Figure 3.9 shows the spheroid after one day treatment. Hoechst 33258 is a fluorescent dye that stains the nucleii of all cells, while propidium iodide (PI) on the other hand only stains nucleii from dead cell. The fluorescence can then be used to quantify and

the ratio of fluorescence of PI to Hoechst calculated, to give the cell viability (Figure 3.10). As we can see from Figures 3.9 and 3.10, ATZ loaded micelles are significantly more effective at killing tumor cells than ATZ alone for the same concentration. Furthermore, ATZ alone at the used concentration is ineffective which clearly demonstrates the efficiency of the drug delivery system. It is important to note that empty micelle does not have any significant effect by itself which proves the non-toxicity of the drug delivery system developed. This study shows that after only one day treatment with ATZ loaded micelle the cell viability is drastically reduced.

3.3 - Conclusions

Self-assembly of linear dendritic block copolymers of various ratios was studied using a co-solvent evaporation method. Spherical micelles were obtained from G1PEG2000 and G2PEG2000 while rod-like structures from G2PEG6000. The self-assembly of G1PEG2000 micelle was further explored in response to change in pH. TEM monitoring of the pH stimuli brought a new insight in the micellar behaviour and revealed swelling and shape-shifting of the micelle followed by aggregation of the nanostructure. This behavior expands our understanding of such LDBC in terms of their response to external stimulus. Finally, the biological studies on cancer cell spheroids revealed the great potential of the developed LDBC drug delivery.

3.4 - Experimental

Preparation of micelles

The self-assembly studies of LDBC were carried out using a co-solvent evaporation method. In a typical procedure, specific amount (0.15-1.5 mg/mL) of LDBC was dissolved in 1 mL of acetone. The acetone solution was added dropwise (1 drop / 10 seconds) to stirring deionized water (1.5 mL). The mixture was stirred in the dark overnight to remove acetone and trigger micelle formation. The aqueous solution was filtered through a 0.45 μm Sterlitech PVDF filter to remove any dust.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) measurements were performed on a NanoBrook 90Plus Particle Size Analyzer equipped with an Brookhaven's TurboCorr correlator (ALV GmbH), a 35mW red diode laser (λ = 640 nm). The scattered light was measured at an angle of 90°, and at a temperature of 25°C. A cumulant analysis was applied to obtain the diffusion coefficient (D) of micelle in solution. The hydrodynamic radius (RH) of micelles were obtained using the Stokes-Einstein equation. The constrained regularized CONTIN method was used to obtain the particle size distribution. Samples were filtered through a 0.45 μm Sterlitech PVDF membrane prior to measurements. The data presented are the mean of three measurements ± S.D.

TEM sample preparation

The carbon-coated 400 square-mesh copper grids (CF400-Cu) were negatively charged prior to addition of a drop (7 μL) of the micellar solution (0.1 mg/mL) sample, and left on it for 30 seconds. A drop (7 μL) of the staining solution was deposited on the grid and left on the grid for 1 minute. The sample was allowed to dry overnight at room temperature.

TEM

Transmission electron microscopy (TEM) was used to capture images of the micelles using a Tecnai T12 electron microscope equipped with an AMT XR 80C CCD camera at an acceleration voltage of 120 kV.

pH studies

The buffer solution was prepared from acetic acid and sodium acetate solution. An aliquot of the micellar solution (0.3 mL) was added to the buffer solution (1.7 mL), and the pH verified. DLS and TEM sample preparation were performed at the various reported time.

Critical Micelle Concentration (CMC) determination

Given volumes of pyrene stock solution in acetone (180 μM) were added to a series of 4 mL vials and the acetone was allowed to evaporate overnight in the dark. Blank LDBC micelles were prepared following the general procedure described above. Specified volumes of the micellar solutions were added to the vials having pyrene so that micelle concentration varied from 0.01 to 200 μg/mL while pyrene concentration was kept constant at 6 μM. The mixture was stirred overnight in the dark. Pyrene fluorescence excitation spectra were recorded from 360 to 410 nm following at emission of 390 nm. The ratios of

the first/third pyrene vibronic peaks (I1/I3) were plotted versus polymer concentration. Steady-state fluorescence spectra were recorded using a Varian Cary Eclipse fluorescence spectrophotometer.

Drug loading

Various amounts of drug were loaded inside the micelle. It was done by diluting different amounts of ATZ with the LDBC acetone solution and following the procedure for blank micelle preparation. The ATZ concentration was determined by HPLC analysis of acetazolamide (ATZ). It was performed on an Agilent Technologies HP 1260 infinity chromatography system equipped with a quaternary pump, a UV-visible diode array detector, a column thermostat and a HP Vectra computer equipped with the HP-Chemstation software. The assay was carried out at 25 ºC using a 250x4.6 mm column filled with 2.7 µm-reversed phase EC-C18 Agilent eluted at a flow rate of 1.0 mL/min with 100% methanol. The injection volume was 10 µL and the run time was 5.0 min. ATZ, monitored by its absorbance at 237 nm, had a retention time of about 0.23 min. A calibration curve ($r2 \geq 0.999$) of acetazolamide was prepared using standard solutions ranging in concentration from 10 to 50 µg/mL prepared immediately prior to the assay. To assay ATZ content of different LDBC micelles, a given volume of the micellar solution was dissolved in methanol to reach micellar concentration of 0.1 mg/mL. A given weight of the polymer alone was suspended in the same solvent mixture (0.1 mg/mL), filtered and used as a control. ATZ encapsulation efficiency and loading efficiency were calculated equations 1 and 2.

Biological Studies

Morphological changes in U251N spheroids upon treatment with ATZ micelles (1 day). U251N spheroids (5K) were developed in a 96-well plate coated with 2% agarose (Invitrogen) in serum-deprived DMEM solution. Spheroids were seeded and maintained in filtered (0.22 μm) complete DMEM medium for four days followed by drug treatments with the empty micelles, ATZ (100uM), ATZ micelles (100uM). PI and Hoechst 33342 fluorescent dyes were added 4h prior to measurements. Following treatment, individual spheroids were imaged using a fluorescent microscope. Significant cell death caused by ATZ-micelles. Following treatments, PI and Hoechst 33342 fluorescent dyes were added 4h prior to measurements. Spheroids were then carefully transferred onto a microscope slide using a pipette, and flattened under a coverslip. Imaging after flattening of the spheroids was conducted using fluorescence microscope, and fluorescence intensity was quantified using ImageJ software. The ordinate shows the relative PI to Hoechst-33342 fluorescent intensity. The abscissa shows the concentration of the drugs as indicated. Average values and S.E.M.s are reported for three measurements which were repeated in at least two independent experiments. Statistically significant differences from control were calculated using a t-test and are indicated by * ($p<0.05$).

3.5 - References

1. Simonyan, A.; Gitsov, I., Linear-Dendritic Supramolecular Complexes as Nanoscale Reaction Vessels for "Green" Chemistry. Diels−Alder Reactions between Fullerene C60 and Polycyclic Aromatic Hydrocarbons in Aqueous Medium. *Langmuir* **2008,** *24* (20), 11431-11441.
2. Savić, R.; Luo, L.; Eisenberg, A.; Maysinger, D., Micellar Nanocontainers Distribute to Defined Cytoplasmic Organelles. *Science* **2003,** *300* (5619), 615-618.
3. Pochan, D. J.; Chen, Z.; Cui, H.; Hales, K.; Qi, K.; Wooley, K. L., Toroidal Triblock Copolymer Assemblies. *Science* **2004,** *306* (5693), 94-97; Ott, C.; Hoogenboom, R.;

Hoeppener, S.; Wouters, D.; Gohy, J.-F.; Schubert, U. S., Tuning the morphologies of amphiphilic metallo-supramolecular triblock terpolymers: from spherical micelles to switchable vesicles. *Soft Matter* **2009,** *5* (1), 84-91.
4. Paleos, C. M.; Tsiourvas, D.; Sideratou, Z.; Tziveleka, L. A., Drug delivery using multifunctional dendrimers and hyperbranched polymers. *Expert opinion on drug delivery* **2010,** *7* (12), 1387-98.
5. Sousa-Herves, A.; Riguera, R.; Fernandez-Megia, E., PEG-dendritic block copolymers for biomedical applications. *New J. Chem.* **2012,** *36* (2), 205-210.
6. Gitsov, I.; Lambrych, K. R.; Remnant, V. A.; Pracitto, R., Micelles with highly branched nanoporous interior: Solution properties and binding capabilities of amphiphilic copolymers with linear dendritic architecture. *J. Polym. Sci. A Polym. Chem.* **2000,** *38* (15), 2711-2727.
7. Cheng, L.; Cao, D., Effect of Tail Architecture on Self-Assembly of Amphiphiles for Polymeric Micelles. *Langmuir* **2009,** *25* (5), 2749-2756.
8. Gitsov, I.; Wooley, K. L.; Fréchet, J. M. J., Novel Polyether Copolymers Consisting of Linear and Dendritic Blocks. *Angew. Chem. Int. Ed. in English* **1992,** *31* (9), 1200-1202.
9. Javakhishvili, I.; Hvilsted, S., Biochemical Nanomaterials Based on Poly(ϵ--caprolactone). In *Organic Nanomaterials*, John Wiley & Sons, Inc. 2013; pp 79-101.
10. Lundberg, P.; Walter, M. V.; Montanez, M. I.; Hult, D.; Hult, A.; Nystrom, A.; Malkoch, M., Linear dendritic polymeric amphiphiles with intrinsic biocompatibility: synthesis and characterization to fabrication of micelles and honeycomb membranes. *Polym. Chem.* **2011,** *2* (2), 394-402.
11. Gillies, E. R.; Jonsson, T. B.; Fréchet, J. M. J., Stimuli-Responsive Supramolecular Assemblies of Linear-Dendritic Copolymers. *J. Am. Chem. Soc.* **2004,** *126* (38), 11936-11943.
12. Kalva, N.; Aswal, V. K.; Ambade, A. V., Effect of the Branching Pattern of Hydrophobic Dendrons on the Core Structure of Linear-Dendritic Copolymer Micelles. *Macromol. Chem. Phys.* **2014,** *215* (15), 1456-1465.
13. van Hest, J. C. M.; Delnoye, D. A. P.; Baars, M. W. P. L.; van Genderen, M. H. P.; Meijer, E. W., Polystyrene-Dendrimer Amphiphilic Block Copolymers with a Generation-Dependent Aggregation. *Science* **1995,** *268* (5217), 1592-1595.
14. del Barrio, J.; Oriol, L.; Sánchez, C.; Serrano, J. L.; Di Cicco, A.; Keller, P.; Li, M.-H., Self-Assembly of Linear−Dendritic Diblock Copolymers: From Nanofibers to Polymersomes. *J. Am. Chem. Soc.* **2010,** *132* (11), 3762-3769.
15. Israelachvili, J. N.; Mitchell, D. J.; Ninham, B. W., Theory of self-assembly of hydrocarbon amphiphiles into micelles and bilayers. *J. Chem. Soc. Faraday Trans. 2: Molecular and Chemical Physics* **1976,** *72* (0), 1525-1568.
16. Stuart, M. A. C.; Huck, W. T. S.; Genzer, J.; Muller, M.; Ober, C.; Stamm, M.; Sukhorukov, G. B.; Szleifer, I.; Tsukruk, V. V.; Urban, M.; Winnik, F.; Zauscher, S.; Luzinov, I.; Minko, S., Emerging applications of stimuli-responsive polymer materials. *Nat Mater* **2010,** *9* (2), 101-113.
17. Jeong, B.; Gutowska, A., Lessons from nature: stimuli-responsive polymers and their biomedical applications. *Trends Biotechnol.* **2002,** *20* (7), 305-311.

18. Skirtach, A. G.; Muñoz Javier, A.; Kreft, O.; Köhler, K.; Piera Alberola, A.; Möhwald, H.; Parak, W. J.; Sukhorukov, G. B., Laser-Induced Release of Encapsulated Materials inside Living Cells. *Angew. Chem. Int. Ed.* **2006,** *45* (28), 4612-4617.
19. Morimoto, N.; Qiu, X.-P.; Winnik, F. M.; Akiyoshi, K., Dual Stimuli-Responsive Nanogels by Self-Assembly of Polysaccharides Lightly Grafted with Thiol-Terminated Poly(N-isopropylacrylamide) Chains. *Macromolecules* **2008,** *41* (16), 5985-5987.
20. Kreft, O.; Javier, A. M.; Sukhorukov, G. B.; Parak, W. J., Polymer microcapsules as mobile local pH-sensors. *J. Mater. Chem.* **2007,** *17* (42), 4471-4476.
21. Blasco, E.; Piñol, M.; Oriol, L., Responsive Linear-Dendritic Block Copolymers. *Macromol. Rapid Commun.* **2014,** *35* (12), 1090-1115.
22. Shi, Z.; Lu, H.; Chen, Z.; Cheng, R.; Chen, D., Rational design, syntheses, characterization and solution behavior of amphiphilic azobenzene-containing linear-dendritic block copolymers. *Polymer* **2012,** *53* (2), 359-369; Blasco, E.; Barrio, J. d.; Piñol, M.; Oriol, L.; Berges, C.; Sánchez, C.; Alcalá, R., Azobenzene-containing linear–dendritic block copolymers prepared by sequential ATRP and click chemistry. *Polymer* **2012,** *53* (21), 4604-4613.
23. Urdabayev, N. K.; Popik, V. V., Wolff Rearrangement of 2-Diazo-1(2H)-Naphthalenone Induced by Nonresonant Two-Photon Absorption of NIR Radiation. *J. Am. Chem. Soc.* **2004,** *126* (13), 4058-4059.
24. Sun, L.; Zhu, B.; Su, Y.; Dong, C.-M., Light-responsive linear-dendritic amphiphiles and their nanomedicines for NIR-triggered drug release. *Polym. Chem.* **2014,** *5* (5), 1605-1613.
25. Ge, Z.; Luo, S.; Liu, S., Syntheses and self-assembly of poly(benzyl ether)-b-poly(N-isopropylacrylamide) dendritic–linear diblock copolymers. *J. Polym. Sci. A Polym. Chem.* **2006,** *44* (4), 1357-1371.
26. Alarcon, C. d. l. H.; Pennadam, S.; Alexander, C., Stimuli responsive polymers for biomedical applications. *Chem. Soc. Rev.* **2005,** *34* (3), 276-285.
27. Zhang, Y.; Xiao, C.; Li, M.; Chen, J.; Ding, J.; He, C.; Zhuang, X.; Chen, X., Co-delivery of 10-Hydroxycamptothecin with Doxorubicin Conjugated Prodrugs for Enhanced Anticancer Efficacy. *Macromol. Biosci.* **2013,** *13* (5), 584-594.
28. Xiang, Y.; Ma, B.; Li, T.; Yu, H. M.; Li, X. J., Acetazolamide suppresses tumor metastasis and related protein expression in mice bearing Lewis lung carcinoma. *Acta pharmacologica Sinica* **2002,** *23* (8), 745-51.
29. Mai, Y.; Eisenberg, A., Self-assembly of block copolymers. *Chem. Soc. Rev.* **2012,** *41* (18), 5969-5985.
30. Rodríguez-Hernández, J.; Chécot, F.; Gnanou, Y.; Lecommandoux, S., Toward 'smart' nano-objects by self-assembly of block copolymers in solution. *Prog. Polym. Sci.* **2005,** *30* (7), 691-724.
31. Ohi, M.; Li, Y.; Cheng, Y.; Walz, T., Negative staining and image classification — powerful tools in modern electron microscopy. *Biol. Proced. Online* **2004,** *6* (1), 23-34.
32. Yue, J.; Liu, S.; Xie, Z.; Xing, Y.; Jing, X., Size-dependent biodistribution and antitumor efficacy of polymer micelle drug delivery systems. *J. Mater. Chem. B* **2013,** *1* (34), 4273-4280.
33. Wurm, F.; Frey, H., Linear–dendritic block copolymers: The state of the art and exciting perspectives. *Prog. Polym. Sci.* **2011,** *36* (1), 1-52.

34. Allen, C.; Maysinger, D.; Eisenberg, A., Nano-engineering block copolymer aggregates for drug delivery. *Colloids and Surfaces B: Biointerfaces* **1999,** *16* (1–4), 3-27.
35. Francis, M. F.; Lavoie, L.; Winnik, F. M.; Leroux, J.-C., Solubilization of cyclosporin A in dextran-g-polyethyleneglycolalkyl ether polymeric micelles. *European Journal of Pharmaceutics and Biopharmaceutics* **2003,** *56* (3), 337-346.
36. Dominguez, A.; Fernandez, A.; Gonzalez, N.; Iglesias, E.; Montenegro, L., Determination of Critical Micelle Concentration of Some Surfactants by Three Techniques. *J. Chem. Educ.* **1997,** *74* (10), 1227.
37. Nasiru, T.; Avila, L.; Levine, M., Determination of critical micelle concentration using UV visible spectroscopy. *Journal of high school research* **2011,** *2*, 1-5.

Chapter 4: Conclusions

4.1 - Summary and conclusions

Linear dendritic block copolymers (LDBC) are a novel class of macromolecules with potential for numerous applications. As in linear diblock copolymers, amphiphilic LDBC provide opportunities to self-assemble into a variety of nanostructures. The dendritic components can impart enhanced stability to the self-assembled structures and allow better loading of drug molecules inside the self-assembled architecture, compared to linear diblock copolymers, which is an added advantage for drug delivery.

A series of LDBC containing bis-MPA dendron and PEG chain were prepared in this study. To further enhance controlled delivery of drug molecules, the surface of the dendrons was functionalized with pH sensitive acetonide groups. The divergent synthesis of bis-MPA dendrons was achieved using highly efficient chemistry and led to the development of generations 1 to 3. The generations 1 and 2 dendrons were successfully coupled to the pentaerythritol core via Cu(I) alkyne azide cycloaddition. The LDBCs were prepared by the coupling method between the dendrons and two different PEG chains. The new macromolecules were fully characterized with ^{1}H- ^{13}C-NMR spectroscopy, MS, and GPC.

Self-assembly of LDBC was studied using co-solvent evaporation method, and the resulting structures were analysed by DLS and TEM. Monodisperse spherical micelles of approximately 150-200 nm were obtained from G1PEG2000 and G2PEG2000 and larger rod-like structures from G2PEG6000. The pH responsiveness of the self-assembled LDBC

was examined using DLS and TEM technique, and showed swelling and aggregation of G1PEG2000 micelles. The unusual response was monitored by TEM, which has allowed to image the shape-shifting effect of pH, giving a better insight in the pH response of this type of micelles.

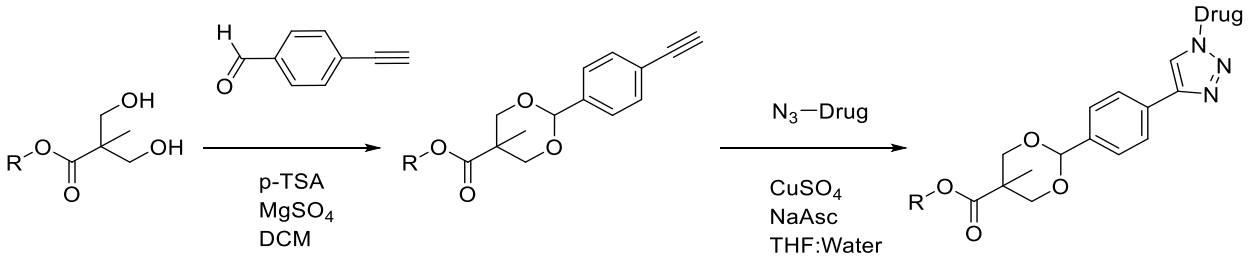
Drug delivery capabilities of these self-assembled structures from LDBC was examined by loading acetazolamide into micelles and studying its efficacy *in vitro*. The blank micelles did not show any cytotoxicity, but for similar concentration, the loaded drug delivery system showed more toxicity towards cancer cells than ATZ alone. It is the first time ATZ was encapsulated inside micellar LDBC and tested for drug delivery, and this work shows the great potential micellar LDBC holds.

We have developed a simple and highly versatile methodology to synthesize LDBC, dendrons are prepared using efficient chemistry, and can be simply coupled to linear polymers. Furthermore, pH sensitive groups were used to decorate the dendron and they could easily be elaborated to include any other molecules at their peripheries. Our results show that their self-assembly is dependent on the composition of the LDBC, and one could obtain from micelle to rod like shapes in these nanostructures. The pH responsiveness of the system demonstrates that these LDBC could help tailor drug delivery at desired locations. The loading efficiency of drugs into self-assembled structures is high, and provides a method to deliver highly potent drugs to cancer cells. These systems could be easily extended to include any other drug molecules of choice.

4.2 - Future work and outlook

Co-delivery of two different drugs holds tremendous potential for cancer therapy, as the synergy between drugs could be exploited to increase the therapeutic effect. The

advantage of using a drug delivery system for this application is to ensure the release of the two drugs at the same location, at the same time. The surface of the LDBC could be functionalized with one drug while the other will be encapsulated inside the self-assembled system. To keep the pH responsiveness of the system, an alternative surface group is needed. One based on acetal formation is proposed in Scheme 4.1.



Scheme 4.1 – Alternative functionalization of bis-MPA dendron for combination therapy

We further envisage to understand other aspects of our systems such as the micelle stability, the drug delivery mechanism and the kinetic profile of the drug release. CMC will be determined using new molecular probes. The kinetic profile of drug release can be determined by dialysis and may allow better understanding of the drug interaction with the micellar core. Furthermore, comparing the kinetic profile of drug release at pH 7.4 and pH 5 would also give precious information.

Micelles from G2PEG6000 LDBC have a significantly different shape but a similar molecular scaffold to G1PEG2000. Comparing drug delivery results for these two systems will also be of great interest. Finally, *in vivo* studies of the G1PEG2000 micelles would be a good follow up to the *in vitro* experiment, and could reveal the full potential of the drug delivery system.