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Synthesis and characterization of novel temperature-responsive dendritic PEG-PDLLA star polymers for drug delivery

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SYNTHESIS AND CHARACTERIZATION OF NOVEL TEMPERATURE-RESPONSIVE DENDRITIC PEG-PDLLA STAR POLYMERS FOR DRUG DELIVERY

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Engineering at Virginia Commonwealth University.

by

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I would like to dedicate this research work to my entire family. I am grateful to each one of them for their unique methodology of showering love and affection on me. A special note to my father: I will keep working hard and will put in more effort to reach my ultimate goal.

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Abstract

SYNTHESIS AND CHARACTERIZATION OF A NOVEL TEMPERATURE-RESPONSIVE DENDRITIC PEG-PDLLA STAR POLYMERS FOR DRUG DELIVERY

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Engineering at Virginia Commonwealth University.

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This study describes a novel thermoresponsive dendritic polyethylene glycol-poly(D, L-lactide) (PEG-PDLLA) core-shell nanoparticle with potential for drug delivery and controlled release. A series of dendritic PEG-PDLLA nanoparticles were synthesized through conjugation of PEG to Starburst™ polyamidoamine (PAMAM) dendrimer G3.0 and subsequent ring-opening polymerization of DLLA, in which PEG chain length (i.e., MW=1500, 6000 or 12000 Dalton) was varied; however, the feeding molar ratio of DLLA monomers to the overall PEG repeat units on the dendrimer surface was kept at 1:1. Linear PEG-PDLLA copolymers were also synthesized under the same condition and used as

control. According to our results, dendritic PEG-PDLLA in aqueous phase could self-assemble into spherical aggregates and the size of spherical aggregates increased with PEG chain length increase. Further, spherical aggregates made of dendritic PEG-PDLLA exhibited magnified temperature-dependence in terms of solubility change and dimension expansion as compared to linear PEG-PDLLA. The most significant size expansion was observed in particles made of dendritic PEG (12000)-PDLLA, which was twice as much as that of particles made of linear PEG (12000)-PDLLA. Water insoluble antitumor drug camptothecin (CPT) was used as a model drug for encapsulation and release studies. Spherical aggregates encapsulated more CPT when dendritic PEG-PDLLA had a longer PEG-PDLLA chain and/or when temperature was elevated to body temperature. This study demonstrated that nanoscale clustering PEG-PDLLA through dendrimers magnified the thermo-sensitivity of PEG-PDLLA. Successful development of such a new particulate system made of dendritic PEG-PDLLA with an expandable dimension in response to temperature change generated a new direction for designing stimuli-responsive materials.

CHAPTER 1 Introduction

The field of controlled drug delivery acts as a driving force for current innovations in biomaterials. Over the recent years, there has been a lot of advances in the field of drug delivery. Specifically, many types of synthetic and natural polymers have been synthesized and employed as drug delivery vehicles. For most drug delivery systems, polymers function simply as inert, biocompatible carriers to deliver drugs. Recently, polymers with targeting and pathology-responsive functions have drawn considerable attention [1]. Such polymers can be categorized into a broad category termed "stimuli – responsive" materials [2]. The use of such stimuli-responsive materials offers exciting new opportunities with respect to numerous applications, particularly, in biomedical fields such as controlled drug delivery. The underlying principle is that applying an external stimulus (e.g., temperature, pH, ionic strength, etc.) to an intelligent system could trigger the release of active agents, which is very helpful in controlling the release pattern of drugs.

Out of the enormous number of synthetic and natural polymers, poly (ethylene glycol) (PEG) and poly (lactic acid) (PLA) block copolymers have emerged as promising biodegradable materials due to their highly controllable chemical and physical properties as well as their favorable biological properties. A lot of studies have been done with PEG-PLA in the past [3]. Hubbell's studies demonstrated changes in the properties of PEG and

PLA by adjusting the respective polymer's sizes in the polymer network [4]. They also demonstrated the specific roles of each block (i.e. of PEG and PLA separately) in the PEG-PLA copolymer. The presence of two polymer blocks enables the system to capture the advantages of both PEG and PLA [5]. PEG groups are hydrophilic and act as channel to bring water into the system. The presence of PEG in the block copolymer prevents non-specific binding of proteins and helps in controlling various biomaterial-cell interactions while PLA blocks are less hydrophilic than PEG and enable the biodegradability of the material with their hydrolytically cleavable ester moieties[6, 7]. Further, PEG-PLA is thermoresponsive i.e., it responds to change in temperature. Though PEG-PLA diblock copolymers have been used in drug delivery, there are some factors which limit this capability. The current status of the PEG-PLA diblocks is that the conformation of PEG blocks at the PEG-PLA nanoparticle surface is yet to be addressed [8]. Hence there is a need of a new approach to increase the efficiency of drug release and encapsulation [2].

The aim of this thesis was to create a novel thermo-responsive drug delivery system using polyamidoamine (PAMAM) dendrimers conjugated with PEG-PLA and to compare the drug encapsulation and release characteristics with the PEG-PLA di-block copolymer. The thesis also attempted at creating a dendrimer based hydrogel (using PAMAM-PEG-PLA) and to study its characteristics with respect to drug encapsulation and release upon temperature variation. Finally, this project explored the synthesis of a dendrimer based pH-sensitive drug delivery system by conjugating PAMAM-PEG to poly (asparatic) acid (PAA).

CHAPTER 2 Background

2.1 Polyethylene Glycol (PEG)

Polyethylene glycol (PEG) is an important type of synthetic polyether. PEG is widely used in drug delivery, wound healing, and a variety of other biomedical applications. PEG is produced by the interaction of ethylene oxide with water and ethylene in presence of acidic or basic catalysts. Ethylene glycol and its oligomers allow the creation of polymers with low polydispersity and narrow molecular weight distribution [9]. The length of the polymer chain depends on the ratio of reactants. These polymers are amphiphilic and soluble in water as well as in many organic solvents (e.g., methylene chloride, ethanol, toluene, acetone, and chloroform). Low molecular weight (M_w <1,000) PEGs are viscous and colorless liquids, while higher molecular weight PEGs are waxy, white solids with melting points proportional to their molecular weights to an upper limit of about 67 °C. PEG also varies in geometry from branched PEG having 3 to 10 PEG chains emanating from a central core group to star PEG having 10 - 100 PEG chains emanating from a central core group [10].

PEG is non-toxic, and resists recognition by the immune system. PEG may transfer its properties to another molecule when it is covalently bound to that molecule. This could

result in toxic molecules becoming non-toxic or hydrophobic molecules becoming soluble when covalently bonded with PEG [11].An improved utilization of PEG is through star polymer structures. Star polymers are three-dimensional hyper-branched structures in which linear arms of the same or different molecular weight emanate from a central core. Star polymers may be used in a variety of biomedical and pharmaceutical applications because they provide a high density of functional groups in a small volume [12].

2.2 Poly(Lactic Acid)

Poly (Lactic Acid) (PLA) is a typical biodegradable polyester, which can be produced from renewable resources such as corn or sugarcane [13, 14]. PLA is an enantiomeric polyester including poly(L-lactic acid)(PLLA) and poly(D-lactic acid)(PDLA). The chiral center in the structure allows varied enantiomeric compositions of PLA. With good biocompatibility, biodegradability and processability, PLA has been widely studied as a matrix material for drug delivery [15, 16]. PLA could undergo scission in the body to monomeric units of lactic acid as a natural intermediate in carbohydrate metabolism [17, 18].

2.3 Dendrimers

Dendrimers represent a relatively new class of highly branched polymers with a well-defined structure that allows precise control of size, shape and terminal group functionality. Dendrimers have a lot of advantages that would make them the ideal choice for a nano-scale drug delivery material. Some properties include good biocompatibility, low polydispersity and highly controllable structure [19]. The advantages of dendrimer based drug delivery are

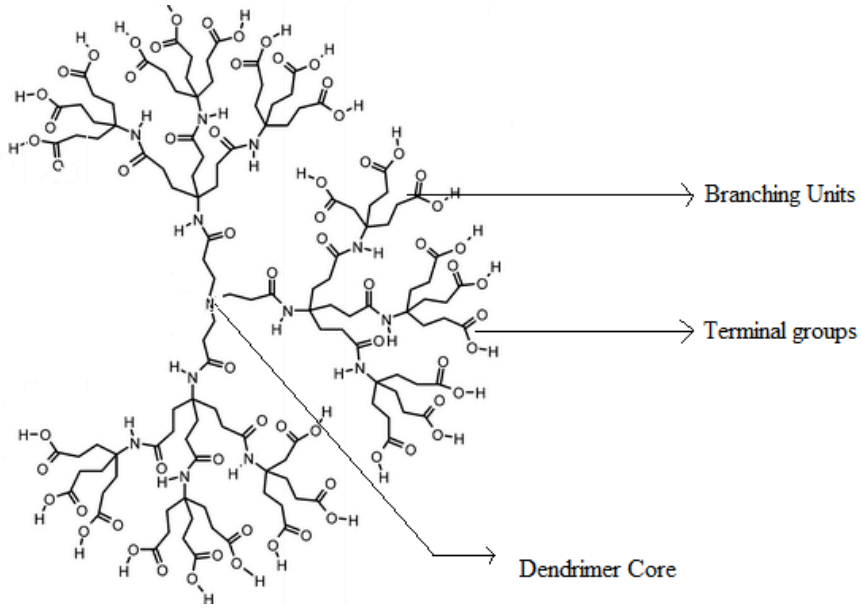
1. Controlled drug release.
2. Dendritic micelles are generally unimolecular and do not suffer from even the low critical micelle concentration (CMC) that the linear polymer based micelles have.
3. They are rapidly internalized into cells through endocytosis due to their nanometer-scale dimensions.
4. Continuously maintain drug levels in a therapeutically desirable range.
5. Decrease amount of drug needed and decrease number of dosages and possibly less invasive dosing and improve patient compliance with the prescribed drug regimen.

As there are a number of surface sites on the dendrimer surface, moieties of various functionalities can be simultaneously attached to the surface through covalent or non-

covalent bonding to make dendrimers multifunctional [19]. A number of studies have been done on dendrimers and their potential applications in drug delivery [20-22].

2.3.1 Structure of Dendrimers

Figure 1. Schematic structure of a dendrimer.



Dendrimers have a well organized structure, which is highly defined by its globular configuration. They consist of a large number of branches, which increase exponentially with generation. These branches initiate from the core and extend all the way to the periphery. The growth and branching of the monomers is counteracted by the steric

hindrance. The branching terminates whenever the steric hindrance is high [23]. In general, a dendrimer molecule consists of three distinct parts – an initiator core, monomers which attach themselves to the core, and terminal functional group [24]. A schematic of a PAMAM dendrimer is showed in Figure 1.

The core of a dendrimer is multi-functionalized and forms the heart of the molecule. The first monomer branches, which emanate from this core, are termed as the "first-generation". For every two monomers that attach to the ends of the previous monomer, the generation increases by 1. As the name indicates, the terminal group is attached to the tail of the monomer [25]. The terminal groups plays an important role in determining the chemical properties of the molecule as well as certain physical properties like viscosity, solubility, etc. As one can expect, the surface area of a dendrimer molecule increases with the number of generations. However, within the molecule, there exists a significant amount of void space. This space consists primarily of channels and cavities. This property of the dendrimer enables certain unique properties like entrapment of foreign molecules including drugs [24].

The shape of the dendrimer depends mainly on its generation. The shape of lower generation dendrimers (0, 1 and 2) can be easily differentiated from higher generations based on the fact that the former have a highly asymmetric shape and have more open structures and compared to the latter. The globular structure of the dendrimer becomes more evident in higher generation dendrimers (Generation 4 or more) as the chains can grow from the core molecule and become more longer and branched [26]. As they extend out

of the periphery, dendrimers become more densely packed and form a close membrane like structure [26].

Dendrimers cannot grow after a certain limit due to clustering and lack of space. The rate of reaction drops suddenly and further reactions of the end groups cannot occur. The dendrimers are said to have reached a critical branched state and this effect is called the "starburst effect". Different types of dendrimers reach this critical state at different generations. In the PAMAM dendrimer, it is observed at the tenth generation. The tenth generation PAMAM contains 6141 monomer units and has a diameter of about 124 Å. The increasing branch density with generation is also believed to have striking effects on the structure of dendrimers. They are characterized by the presence of internal cavities and by a large number of reactive end groups [27].

2.3.2 Synthesis of Dendrimers

Dendrimers are produced in iterative sequence of reaction steps, in which each additional iteration leads to a higher generation dendrimer. Using specifically designed chemical reactions to create dendrimers is one of the best examples of controlled hierarchical synthesis, an approach that allows the creation of complex systems. Poly-amidoamine dendrimers usually contains two types of cores – an ammonia core or an ethylenediamine core (EDA) [28]. Table 1 tabulates the theoretical properties of PAMAM dendrimers based on both cores.

Table 1. Theoretical properties of PAMAM dendrimers [26].

Generation	Ammonia Core		EDA core	
	Molecular Mass	No of terminal Groups	Molecular mass	No of terminal Groups
0	359	3	516	4
1	1043	6	1428	8
2	2411	12	3252	16
3	5147	24	6900	32
4	10619	48	14196	64
5	21563	96	28788	128
6	43451	192	57972	256
7	87227	384	116340	512
8	174779	768	233076	1024
9	349883	1536	466548	2048
10	700091	3072	933492	4096

There are two common methods by which a dendrimer is synthesized - divergent method or convergent method. There exists a basic difference between these two methods. The divergent method was used initially and the convergent method was created as an improvement to this method.

As the name indicates, in the divergent method, the dendrimer grows outwards from a multifunctional core. The first generation dendrimer is then obtained when a monomer molecule, containing a reactive and two dormant groups, reacts with this core [29,

[30]. The terminal group of the resulting molecule is activated for reactions with other monomers. The generation of the dendrimer depends on the number of molecules that attaches itself to the molecule. The main advantage of the divergent method is that, large quantities of dendrimers can be synthesized. However, this method does have several disadvantages such as structural defects as a result of incomplete and side reactions of the terminal groups. In order to prevent this from occurring, a large excess of reagents is needed. Hence, purifying the final product to yield the final product becomes strenuous [29].

In order to overcome the defects in the divergent synthesis method, the convergent method was developed. In the convergent method, the construction of the dendrimer starts from the terminal groups and slowly progresses towards the core [30].The branched polymer arms , called dendrons, are grown initially and when they are large enough, they are attached to a multifunctional core molecule and a dendrimer is synthesized. There are a number of advantages in using the convergent growth method. The convergent method addresses the issue on product purification- one of the disadvantages of the divergent method. Using the convergent method, minimal defects are present in the final product. Also, it becomes possible to introduce subtle engineering into the dendritic structure by precise placement of functional groups at the periphery of the macromolecule [31]. However steric hindrances occur in this method and this causes a difficulty in synthesizing higher generation dendrimers.

Polyamidoamine (PAMAM) dendrimers were the first synthesized dendrimers by Dow Chemicals Company [27]. At the end of each branch there is a free amino group that can react with two methyl acrylate monomers and two ethylenediamine molecules. Each complete reaction sequence results in a new dendrimer generation.

2.3.3 Dendrimer Calculation

The calculations of the molecular weight and other useful quantities about the dendrimer molecules are presented by Tomalia[32 33]. The number of terminal groups is easily calculated as follows:

$$\text{Number of terminal groups} = N_c (N_r)^G$$

Where N_c is the number of branches at the core (core multiplicity); (N_r) is the number of branches on each monomer unit (repeating unit multiplicity); G is the number of generation. The degree of polymerization can be computed using these quantities.

$$\text{Degree of polymerization,} \quad N_c = \frac{N_r^G - 1}{N_r - 1} \text{ and hence,}$$

$$\text{Molar mass, M} = M_c + N_c \left[M_r \left(\frac{N_r^G - 1}{N_r - 1} \right) + M_t N_r^G \right],$$

where, M_c, M_r and M_t are the molecular weights of the core, the repeating monomer and the terminal group respectively [32, 33].

2.3.4 Dendrimers vs. Linear Polymers

Unlike linear polymers, dendrimers are mono-disperse macromolecules. This means that the molecular weight and the size of dendrimers are controllable during the synthesis whereas those of the linear polymers are non-controllable and are random in nature[34, 35]. Hence we can synthesize dendrimers specifically for the application we need whereas synthesis of linear polymers results in molecules of different sizes. Another advantage of dendrimers over linear polymers is that the former possesses certain significantly improved chemical and physical properties due to their structural architecture.

When dissolved in solution, dendrimers tend to form a tightly packed sphere whilst the linear polymers form flexible coils. A significant application of this occurs in the rheological properties. Another unique property of dendrimers lies in its viscosity[36]. Dendrimers possess significantly lesser viscosity than linear polymers. An interesting fact to note is that up to the fourth generation of dendrimers, with increase in molecular weight, the intrinsic viscosity increases. For generations higher than the fourth, the viscosity tends to decrease with increase in molecular weight. This behavior is unique to high generation dendrimers because linear polymers tend to have higher viscosity with increase in molecular weight [37]. Dendrimers are also have higher solubility and miscibility and are more reactive than classical polymers. This can be explained by the presence of many chain ends in a dendrimer and by the nature of the surface groups. A dendrimer possesses

both hydrophobicity and hydrophilicity within the same molecule. Dendrimers terminated in hydrophilic groups are soluble in polar solvents, while dendrimers having hydrophobic end groups are soluble in nonpolar solvents.

Dendrimers have some unique properties because of their globular shape and the presence of internal cavities. The most important one is the possibility to encapsulate guest molecules in the macromolecule interior. Meijer and co-workers trapped small molecules like rose bengal or p-nitrobenzoic acid inside the 'dendritic box' of poly(propylene imine) dendrimer with 64 branches on the periphery [38]. Then a shell was formed on the surface of the dendrimer by reacting the terminal amines with an amino acid (L-phenylalanine) and guest molecules were stably encapsulated inside the box.

Hydrolyzing the outer shell could liberate the guest molecules. The shape of the guest and the architecture of the box and its cavities determine the number of guest molecules that can be entrapped. Meijer's group described experiments in which they had trapped four molecules of rose bengal or eight to ten molecules of p-nitrobenzoic acid in one dendrimer [26, 39].

CHAPTER 3 Experimental Methods and Materials

3.1 Materials

In this research, chemicals of high purity were utilized as received from the suppliers.

Table 2. Materials used

Materials	Abbreviation
Polyethylene glycol diol [OH-PEG-OH] MW 1500,6000,12000	PEG
Starburst™ G3.0 PAMAM dendrimer (20 wt % solution in methanol)	G3.0
D, L lactide monomers	
Camptothecin	
Triethylamine	TEA
4-nitro phenyl chloroformate	4-NPC
Ethyl ether(anhydrous)	
N,N-dimethylformamide	DMF
Acrolyl chloride	
Ethanol(denatured)	
Ninhydrin	
Eosin Y	
Tetrahydrofluran	THF
Deuterium oxide	D_2O
Toluene	
Stannous Octate	Sn(Oct)2
Dichloromethane	DCM
Hydrochloric Acid	HCl
Chloroform	
De-Ionized Water	DI water

3.2 Equipment

Table 3. List of equipment used

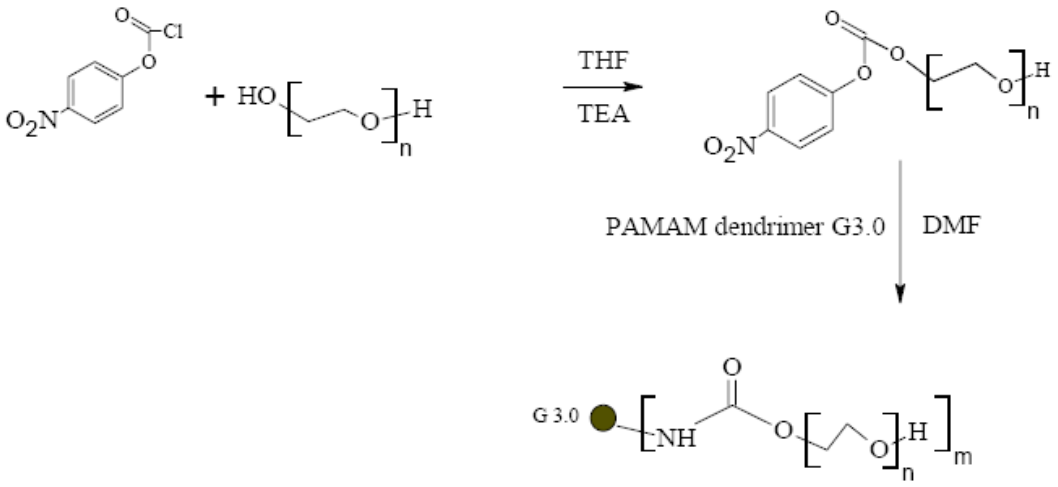
Name	Purpose
Weighing Balance	For measuring the required amount of materials
Eppendorf Centrifuge Model: 5415D	For Centrifugation, separation of the suspended phase form liquid phase.
Ultra Violet – Visible (UV-Vis) Spectrophotometer	Quantitative analytical tool for ninhydrin assay and release experiments.
UV Radiation source	A 90 W high-pressure mercury vapor filled lamp, manufactured by Phillips (Holland), was used as the ultra violet (UV) light source.
Nuclear Magnetic Resonance (NMR) Spectrometer	Proton NMR measurement were carried out on 300 MHz NMR spectrometer
FTS System	Freeze dry system to dry the frozen samples
DLS system (Malvern Zetasizer Nano S)	The Analytical tool for temperature dependent size changes.
FTIR	FTIR spectra was recorded on a Matson Cygnus 100 FTIR spectrophotometer

3.3 Synthesis

3.3.1 Conjugation of PEG to PAMAM dendrimer G3.0

As illustrated in Figure 2, one hydroxyl end group of PEG diol (3 different molecular weights used 1500, 6000 and 12000 Da) was activated first with 4-NPC and TEA to form OH-PEG-NPC conjugates. Briefly 0.4 mmol of PEG was dissolved in 40 ml of THF. To this solution 0.45 mmol (80.6mg) of 4-NPC and 0.4 mmol of TEA were added dropwise. The mixture was stirred for 24 hrs, and then centrifuged at 10 rpm for 10 minutes to filter off the salt. The supernatant was precipitated in ethyl ether (40 ml) and kept at -40 ºC for further precipitation. After 24 hrs, the precipitate was collected and dried using freeze dry system (FTS) to obtain OH-PEG-NPC conjugates. OH-PEG-NPC was then reacted with PAMAM dendrimer generation 3.0 (where the molar ratio of PEG-NPC/dendrimer was 32:1) in DMF for 72 hours forming PEGylated dendrimer conjugate.[28, 40] This solution was precipitated in 50 ml of ethyl ether and kept at -40 ºC for further precipitation. The precipitate was collected and freeze dried with FTS.
Dialysis was carried out to remove excess of PEG for further purification of the product. The resulting G3.0-PEG-OH was then freeze dried. The feeding ratio of OH-PEG-NPC/ dendrimer was maintained at 1:1. The degree of PEGylation on the dendrimer as well as the molecular weight of G3.0-PEG-OH was characterized with ninhydrin assay and ^{1}H-NMR spectroscopy.

Figure 2. Conjugation of PEG to PAMAM dendrimer 3.0 (The feeding molar ratio of OH-PEG-NPC/ dendrimer was maintained at 1:1).



3.3.2 Calculation of amount of lactic acid to be added to PAMAM-PEG.

For a known amount of PAMAM-PEG, The amount of lactic acid (LA) monomers that was to be added was calculated using the following formula,

$$\text{Amount of LA to be added in mg} = \frac{p * x * n * MW_{LA}}{MW_{PAMAM} + (MW_{PEG} * x)}$$

Where,

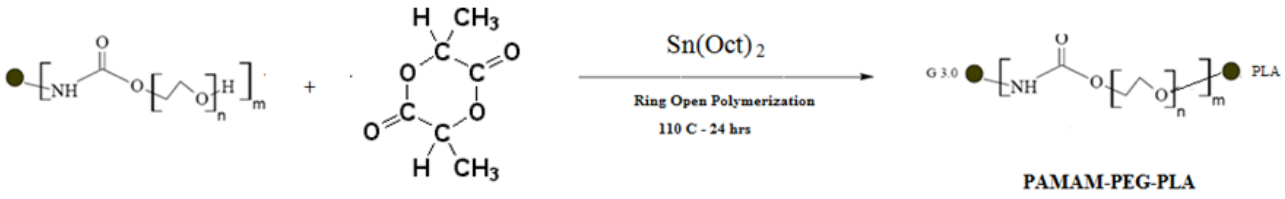
p = Amount of PAMAM-PEG added for conjugation (in mg)
x = Number of PAMAM groups attached to PEG (determined by Ninhydrin assay)
MW = Molecular weight
n = Number of repeat units of PEG = 44.

In order to determine the number of repeat units of PEG, we take a look at the chemical formula of PEG which is (CH_2-CH_2-O). We can determine the molecular weights of this group by knowing that the atomic weights if C, H and O are 12, 1 and 16 approximately. Depending upon the molecular weight of PEG, we can calculate the amount of Lactic acid to be used.

3.3.3 Ring-opening polymerization of D, L-lactide.

G3.0-PEG was then used as the macromonomer to initiate polymerization of D,L-lactide through the hydroxyl end groups of conjugated PEG chains. To a flame dried flask G3.0-PEG, DLLA, 2 mL of toluene and Stannous Octoate (5 µL per 5 g of DLLA) were added. The flask was sealed and stirred at 110°C for 24 hrs to allow ring-opening polymerization of DLLA[41]. Upon completion of the reaction, toluene was evaporated using rotary evaporation. The resulting G3.0-PEG-PDLLA nanoparticles were dissolved in a small volume of DCM and then precipitated in cold diethyl ether, filtered, and vacuum dried. Afterward, extensive dialysis was applied for further purification. A series of dendrimer-based core-shell nanoparticles were synthesized, in which PEG length (i.e., MW=1500, 6000 or 12000 Dalton) was varied, but the molar ratio of DLLA monomers to the PEG repeat units on the dendrimer surface was kept at 1:1. Figure 3 summarizes the synthesis reaction.

Figure 3. Ring Opening polymerization of D,L lactide.



3.3.4 Synthesis of linear PEG-PLA

For comparison, linear PEG (1500)-PDLLA, PEG (6000)-PDLLA, and PEG (12000)-PDLLA copolymers were also synthesized using the same procedure as described above.

3.4 Characterization

3.4.1 Ninhydrin assay

PEGylation degree (i.e., number of PEG per dendrimer) was determined indirectly by measuring the remaining amine surface groups with the ninhydrin assay. The ninhydrin stock solution was prepared by dissolving 70 mg of ninhydrin in 20 mL of ethanol. G3.0-PEG conjugates in very small amounts (1 or 2 mg) were dissolved in 1 mL of ethanol and further mixed with 1 mL of ninhydrin stock solution. The mixture solution was heated to 90 °C for 5 minutes after which it was cooled down to 25ºC. The concentration of amine groups ($[C]_{NH2}$, µmol/mL) was determined at the wavelength of 570 nm based on the

calibration curve, which was established using pure G3.0 PAMAM dendrimer of known concentrations. PEGylation degree (n) was determined by the following equation [42] :

$$[C]_{NH_2} \times V_s = \frac{W_{G3.0-PEG} \times (32 - n)}{MW_{G3.0} + n \times MW_{PEG}} \times 1000$$

where,

V_s = volume of sample solution in the cuvette (ml),

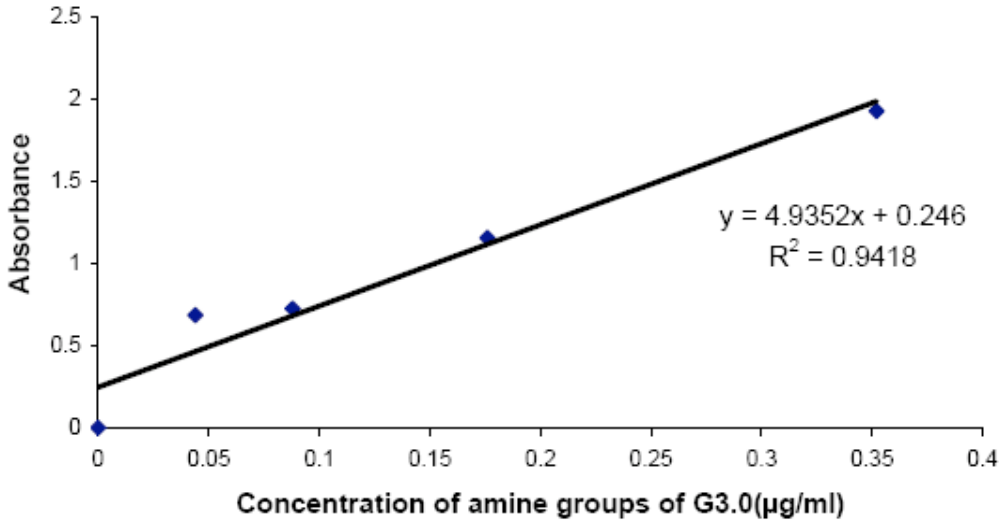
$W_{G3.0-PEG}$ = amount of G3.0-PEG sample used (mg),

$MW_{G3.0}$ = molecular weight of G3.0 PAMAM dendrimer (6909 g/mol),

MW_{PEG} = molecular weight of PEG (i.e., 1500, 6000, or 12000 g/mol)

The calibration curve for a G3.0 PAMAM dendrimer is showed in the figure 4.

Figure 4. Standard curve of a G3.0 PAMAM dendrimer.



3.4.2 ^{1}H-NMR Spectroscopy

^{1}H-NMR spectra of the synthesized polymers were recorded on a Varian superconducting Fourier-transform NMR spectrometer (Inova-400). Deuterium oxide (D_2O, 99.9%) was used as the solvent. The chemical shift for D_2O is 4.8 ppm. Post-processing works were done using the software "Spin-Works". The ^{1}H-NMR spectroscopy test was done to confirm the synthesis and to confirm the PEGylation degree as calculated by the ninhydrin assay test.

The PEGylation degree of the dendrimer was determined based on the integrals of corresponding proton peaks using the formula,

$$\frac{4 * n * X}{m} = \frac{A1}{A2}$$

where :

n = Number of protons in PEG's repeat units (i.e. 44)

m = Number of Protons in PAMAM dendrimer G3.0 (i.e. 476)

X = Degree of PEGylation

A1 = Area under the curve for PEG peak at 3.65ppm

A2 = Relative area under the curve for dendrimer peak between 2.3 ppm and 3 ppm

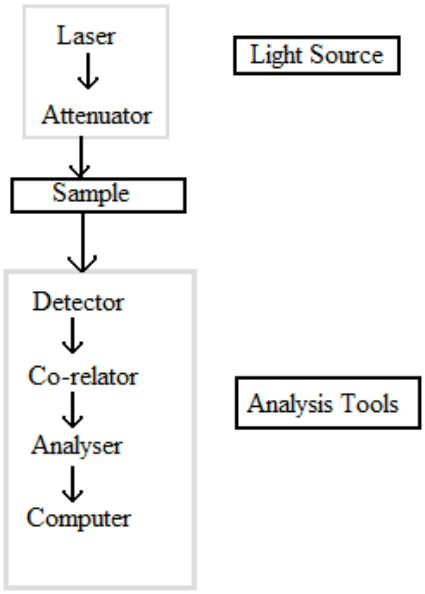
3.4.3 FT-IR

The FT-IR spectra of the synthesized polymers were recorded on a Matson Cygnus 100 FTIR spectrophotometer which is powered by a globular IR source and operates with a Michelson interferometer. The PAMAM-PEG-PLA samples were dissolved in 1ml of methylene chloride, followed by smearing a small quantity of sample solution onto one NaCl plate and then smearing the sample into a film with the second NaCl plate. If a saturated spectrum would result, the NaCl plates were separated, one plate was cleaned with methanol and acetone, and the plates were reassembled for another experiment. No spacer was used. PEG-PLA samples were measured in a similar way as control.

3.4.3 Dynamic Light Scattering

Dynamic Light scattering was used in characterizing the size of the particles (i.e. dendrimer-PEG-PLA and linear PEG-PLA). The sample was prepared at a uniform concentration by dissolving 2mg of the sample in 1 ml of water. Dynamic Light Scattering was performed at 25ºC (±1.5 ºC) and at 37ºC(±1.5 ºC), in order to analyze the size changes. Initially, the particles of PAMAM-PEG-PLA were measured at 25ºC and the temperature was immediately increased to 37ºC(±1.5 ºC) after which the size was noted. Following that, the temperature was brought back to the 25ºC(±1.5 ºC) and the size of the particles were studied and compared with the initial reading. The temperature was again increased to 37ºC(±1.5 ºC) to compare the two readings. This test was performed repeatedly to characterize the size changes of the particles as the temperature varied. In a similar fashion, equivalent concentrations (2mg/ml) of linear PEG-PLA were also studied and the results were compared and contrasted. The schematic of the Dynamic Light Scattering test is shown in the figure 5.

Figure 5. Schematic of the dynamic light scattering (DLS).

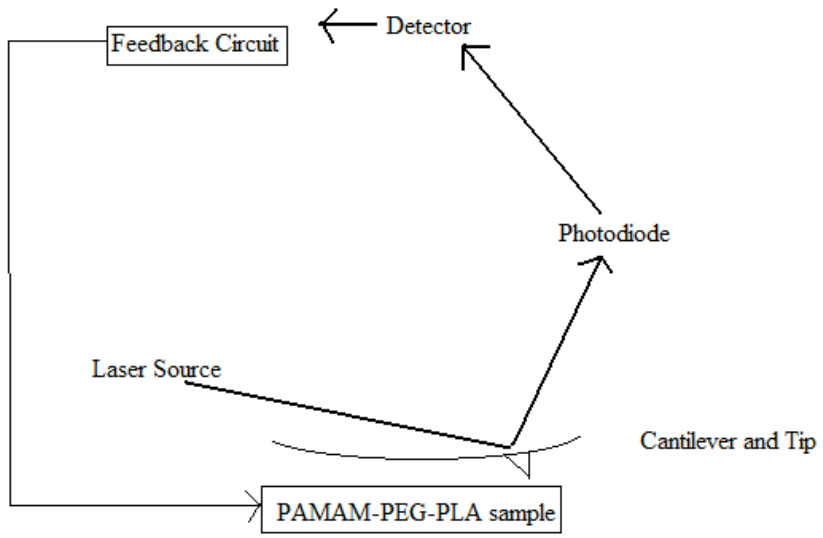


3.4.4 Atomic Force Microscopy (AFM)

The droplet-evaporation method was used for preparing AFM samples (see figure 6). Dendritic PEG-PDLLA nanoparticle sample solutions were prepared and equilibrated at 25ºC and 37ºC (i.e., 37 °C), respectively. A droplet of liquid was deposited on a clean glass slide, which was being maintained at 25ºC or 37ºC. After the sample solution was dried overnight, topographic images of AFM were recorded on a Dimension 3100 (Digital Instruments, Santa Barbara, CA) scanning probe microscope by operating in the tapping mode at 25ºC with a commercial silicon nitride cantilever probe. The probes had a nominal

tip radius ranging from 5 nm to 10 nm and a spring constant in the range from 20 to 40 *N*/m (values provided by manufacturer) (using fundamental resonance frequencies, which ranged between 250 kHz and 350 kHz) for the probe oscillation.

Figure 6. Schematic of atomic force microscopy (AFM).



3.4.5 Solubility Test

The main aim of the solubility tests was to test the solvation of PEG-PAMAM-PLA at various temperatures including 25ºC and 37ºC. In a vial, uniform concentrations of PEG-PAMAM-PLA (60 wt% by weight of PEG-PAMAM-PLA) were taken separately. In order to test the solvation at 25ºC, the vial was vortexed and then allowed to stand still, without stirring for about 2 hours. The degree of salvation of each sample was then recorded. In order to test at 37ºC, a beaker filled with water into the water bath was placed and the temperature of the water bath was set to 37 ºC. After 4-5 hrs, the degree of

salvation of samples was observed. As the degree of nanoparticle solvation at a given temperature was inversely proportional to its UV absorbance value, UV absorbance at the wavelength of 650 nm (visible spectra) was quantified using a UV-Vis spectrometer. Each measurement was repeated three times for accurate analysis. For comparison, solubility of linear PEG-PDLLA at the same concentration was also measured.

3.4.6 Camptothecin (CPT) encapsulation study

Drug Encapsulation studies were done at 25ºC to study the loading capacity of the drug. Camptothecin was used as a model drug in this study. Drug encapsulation was performed using dendritic PEG-PDLLA solutions containing PEG-PDLLA at 2 mmol/mL throughout the study. A constant amount of 10mg of CPT was used in order to determine the encapsulation capabilities. The solution was vortexed and added to a dialysis bag. The set up of the dialysis is depicted in the figure 7. The encapsulation was observed over time by measuring the amount of free drug, the amount of drug that escaped to the beaker and the amount of un-reacted drug. The total drug encapsulated was calculated by using the formula,

$$\text{Total Drug encapsulated} = A_{initial} - A_{free\_drug} - A_{Unreacted}$$

Where,

$A_{initial}$ = Initial amount of drug (i.e. 10mg)

A_{free_drug} = Amount of free drug outside the dialysis bags

$A_{Unreacted}$ = Amount of drug inside the dialysis bags which was not encapsulated.

The amount of CPT having been released was determined by measuring the absorbance of the solution outside the dialysis bag at predetermined intervals. The absorbance of the drug was measured using the UV spectrometer at the wavelength of 315 nm. For comparison, drug release kinetics based on PEG (1500)-PDLLA, PEG (6000)-PDLLA, and PEG (12000)-PDLLA at the same concentration was also studied.

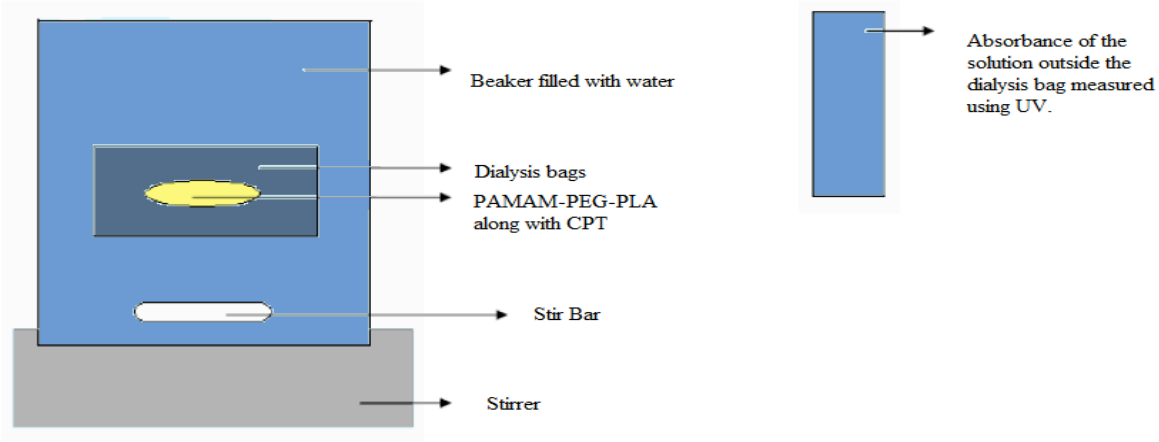
3.4.7 Camptothecin (CPT) Release Study

The release studies were performed at 37ºC in order to mimic the drug kinetics as it happens in the human body. In order to perform the release studies, the drug encapsulated PAMAM-PEG-PLA (4mg of CPT encapsulated in equivalent amounts of PAMAM-PEG-PLA) were taken in dialysis bags; similar to the setup for the encapsulation studies. 4mg was taken as a random sample amount. In order to determine the amount of PAMAM-PEG-PLA that can withhold 4mg of CPT, encapsulation studies were performed, as explained previously. In order to encapsulate 4mg of CPT, 3mmol/ml of PAMAM-PEG-PLA 1500 and 2mmol of PAMAM-PEG-PLA (6000 and 12000) were used. Although 2 mmol of PAMAM-PEG-PLA 6000 and 12000 could encapsulate lot more amount of CPT, When the encapsulation amount showed 4mg of CPT, the experiment was stopped. This method was done to ensure that each sample of PAMAM-PEG-PLA had approximately,

the same amount of CPT encapsulated. Similarly, equivalent amounts of PEG-PLA which could encapsulate 4mg of CPT were also taken. The dialysis bags were placed in a beaker filled with a known volume of water. The temperature of the whole setup was maintained at 37 ºC and a stir bar was used to stir the water in the beaker at a constant rate. On performing dialysis over time, the amount of drug released was calculated by noting the absorbance of the water outside of the dialysis bags and determining the concentration of the CPT drug using UV-spectroscopy. Similar protocols were followed to study the drug release kinetics of linear PEG-PLA.

The drug encapsulation and release kinetics were performed at 25 ºC and 37ºC respectively because these were the temperatures of interest in the study. For example, we were interested in characterizing the drug release at 37 ºC because this is the temperature at which drug will be released in the human body. Drug encapsulation and release kinetics indeed occur at other temperatures as well and are not specific to any temperature, but this was not of interest in performing this study.

Figure 7. Schematic of the drug encapsulation and release kinetics.



CHAPTER 4 Results and Discussion

4.1 Preparation and characterization of dendritic PEG-PDLLA nanoparticles

All the dendrimer surface amine groups were utilized for PEG modification in order to create a saturated PEG layer on the dendrimer surface. PEG-diol was first hetero-functionalized with NPC to make one end hydroxyl group highly reactive toward dendrimer surface amine group. The synthesis procedure was robust and has been validated by our previous work [28, 43]. The yield of G3.0-PEG conjugates based on PEG 1500, PEG 6000, and PEG 12000 was approximately 50%. ^{1}H-NMR spectroscopy confirmed the success of the synthesis (spectra not shown). According to the ninhydrin assay, approximately 23 PEG chains (i.e., PEGylation degree p=23) were attached onto the surface of G3.0, in contrast to the theoretical value of 32. Steric crowding of PEG may impede interaction between ninhydrin and the remaining amine surface groups, leading to inaccurate estimation of PEGylation degree. Alternatively, ^{1}H-NMR was applied to calculate PEGylation degree by integrating appropriate proton peaks from the dendrimer and the conjugated PEG. We found that the PEGylation degree determined by ^{1}H-NMR spectra was comparable to that based on the ninhydrin assay.

After modification of G3.0 with PEG, the hydroxyl groups at the other end of the conjugated PEG chains initiated the ring-opening polymerization of DLLA to produce dendritic PEG-PDLLA core-shell nanoparticles. The yield of dendritic PEG-PDLLA was

approximately 65%. Dendritic PEG-PDLLA nanoparticles were characterized with ^{1}H-NMR. A typical ^{1}H-NMR spectrum of G3.0-PEG (1500)-PDLLA is shown in Figure 8. Peaks **b** and **j** are assigned to the proton in methyl and methane of DLLA, respectively. Peak **h** is assigned to the proton in methylene of the repeat unit of PEG. Peak **c** is assigned to the proton in amide due to conjugation of PEG to primary amine surface group. The rest peaks labeled in the spectrum are assigned to the amide of PAMAM at different locations. For example, according to ^{1}H-NMR spectrum, an average of 6 DLLA monomers (*n*) were polymerized to form a PDLLA block onto each PEG 1500 chain. Dendritic PEG-PDLLA achieved lower polymerization degree of PDLLA than its corresponding linear PEG-PDLLA, due to strong steric crowding effect from the conjugated polymer chains. The composition for all linear and dendritic PEG-PDLLA was calculated based on ^{1}H-NMR and is summarized in Table 4.

Table 4. Composition of linear and dendritic PEG-PDLLA

PEG length (Dalton)	Number of repeat units per PEG (*m*)	Number of repeat unit per PDLLA (*n*)		Molecular weight (*Mn*)	
		Dendritic	Linear	Dendritic	Linear
1500	34	6	16	52265	2652
6000	136	30	41	195223	8952
12000	273	19	87	315661	18264

Note: Molecular weight of dendritic PEG-PDLLA was calculated based on PEGylation degree (i.e. 23) determined by the ninhydrin assay.

Figure 8. ^{1}H -NMR of PAMAM-PEG-PLA 1500

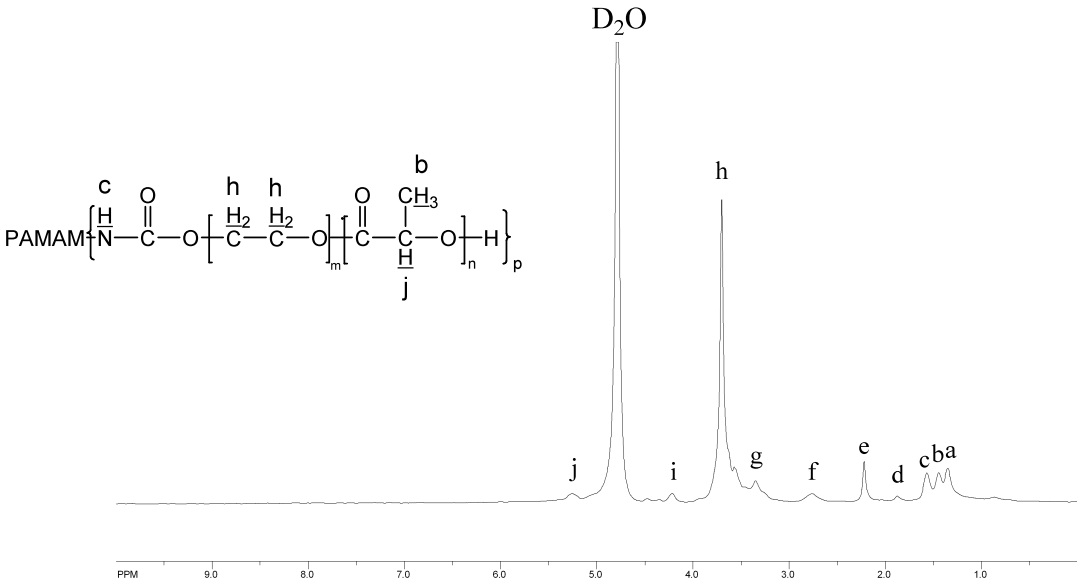


Figure 9. ^{1}H -NMR of PAMAM-PEG-PLA 6000

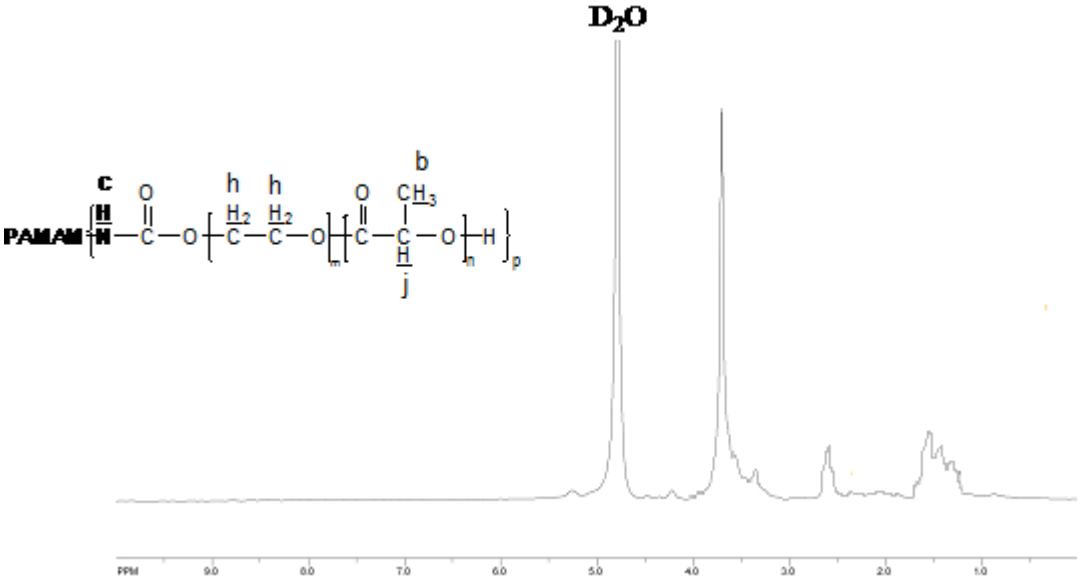


Figure 10. ^{1}H -NMR of PAMAM-PEG-PLA 12000

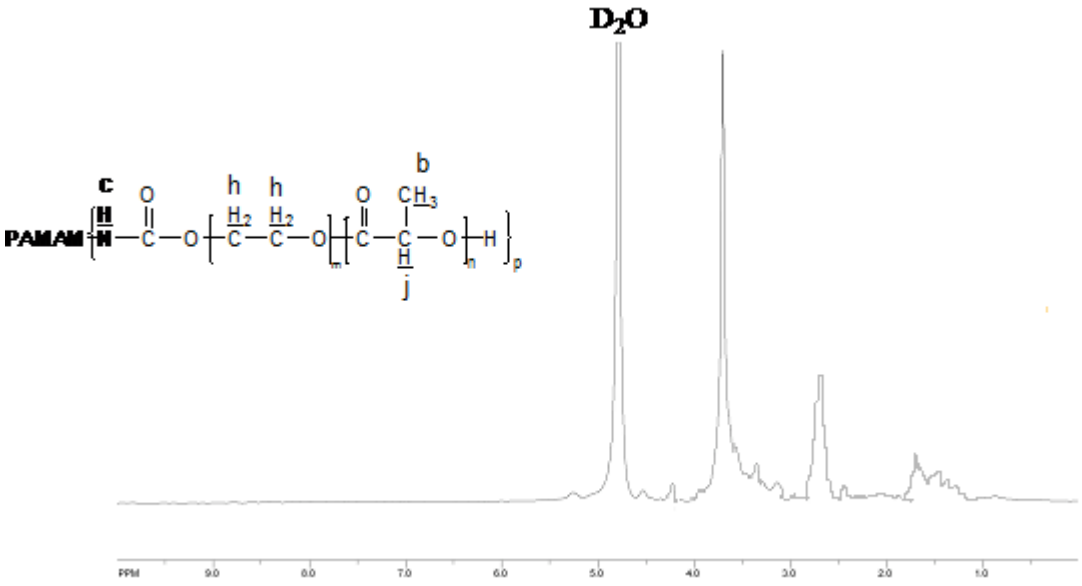


Figure 11. ^{1}H -NMR of PEG-PLA 6000

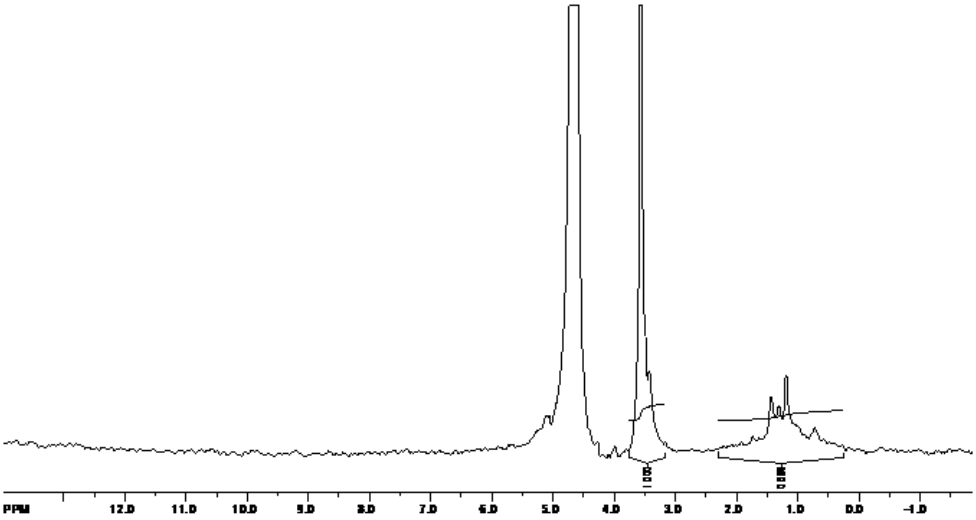
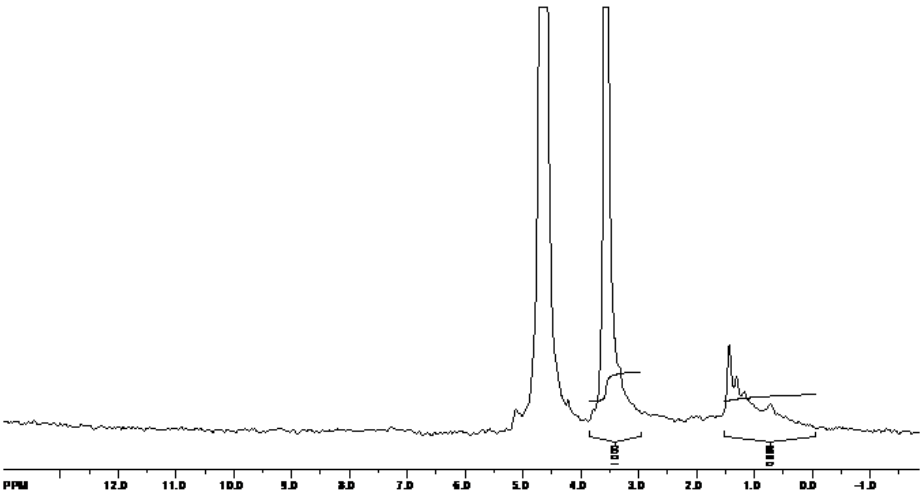


Figure 12. ^{1}H -NMR of PEG-PLA 12000.



4.2 FT-IR results

The FTIR spectra acquired for the PAMAM-PEG-PLA samples were remarkably similar. The area of the region was taken from 500 cm^{-1} to 4000 cm^{-1}. For comparison, the spectra of G3.0 Dendrimer and linear PEG-PLA were taken. From the figures 13, 14 and 15, we can easily confirm the synthesis of PAMAM-PEG-PLA. In figure 13 we find that the spectra between PAMAM-PEG-PLA 6000 and 12000 look remarkably similar. The only difference is the offset in the y-axis. Comparing figure 13 and 14, we can say that the peaks obtained from wavenumbers 500 cm^{-1} to 2000 cm^{-1} belong to that of PEG-PLA, whilst the spectra ranging from 2800 cm^{-1} to 3600 cm^{-1} would belong to that of PAMAM 3.0 Dendrimer. The peak from 2800 cm^{-1} to 3000 cm^{-1} is believed to be the CH_2 group of PAMAM whilst the bigger peak from 3200 cm^{-1} to 3400 cm^{-1} is believed to be that of the NH_2 core.

Figure 13. FTIR spectra of PAMAM-PEG-PLA 6000 and 12000.

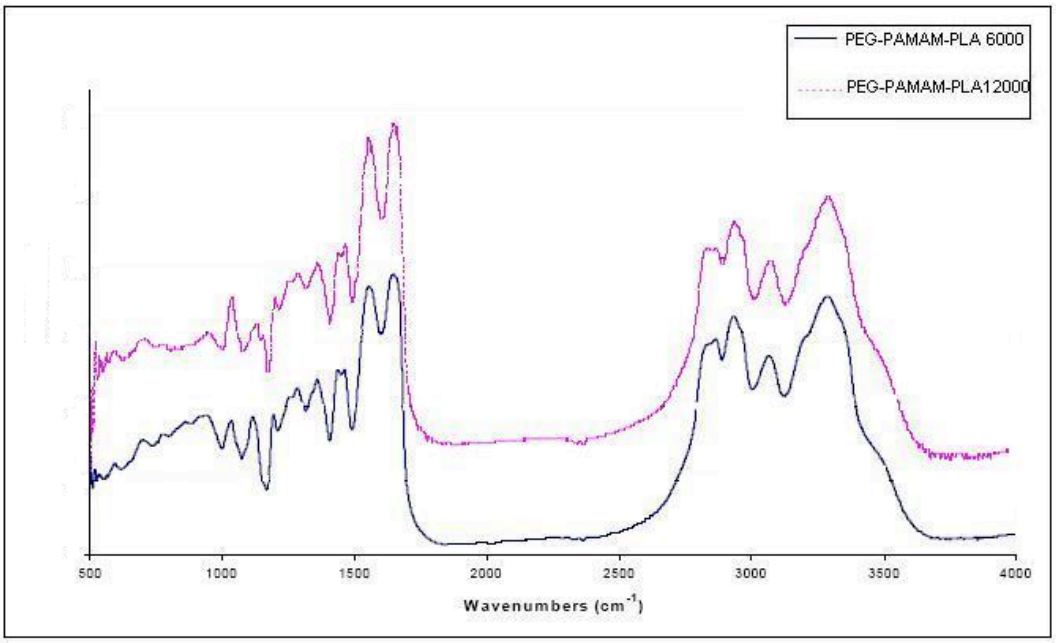


Figure 14. FTIR spectra of PEG-PLA 12000.

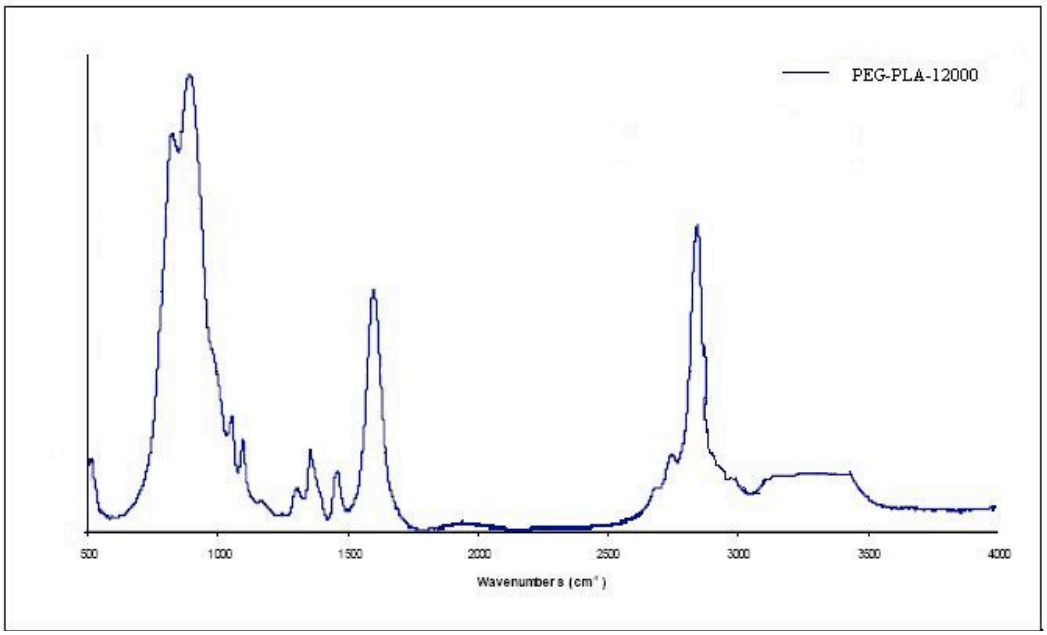
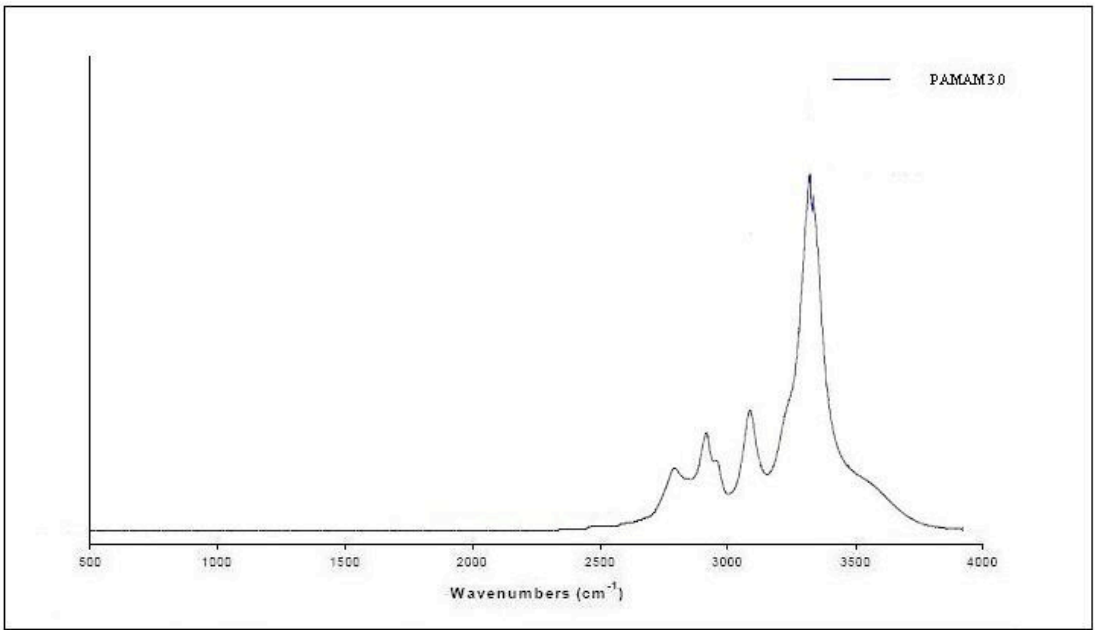


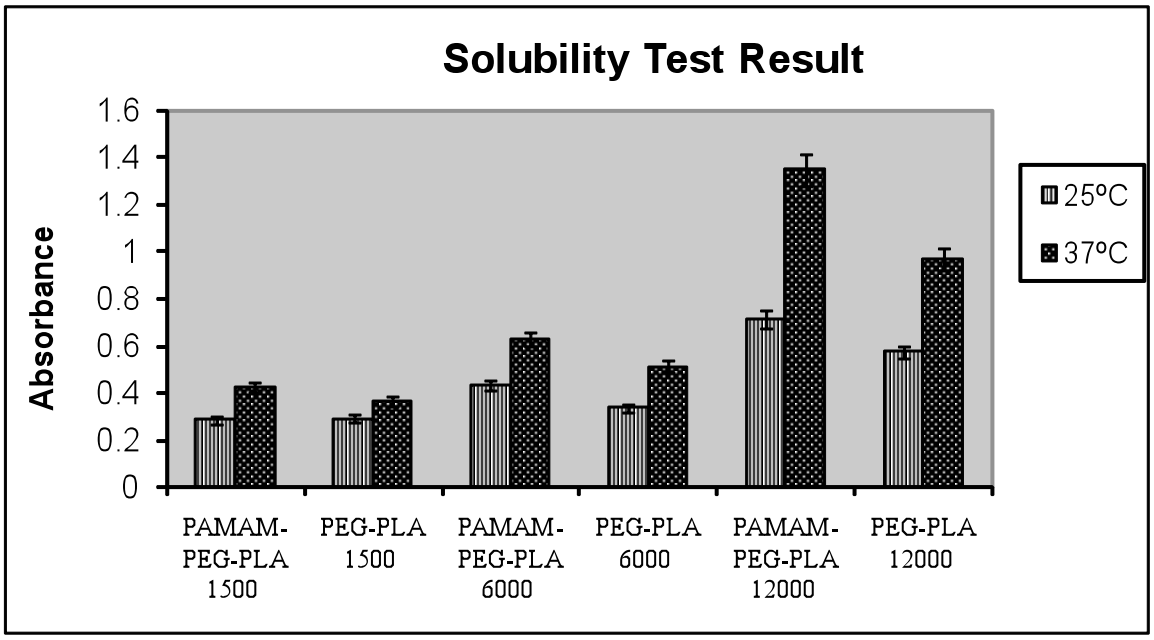
Figure 15. FTIR spectra of PAMAM 3.0.



4.3 Temperature-dependant solubility of dendritic PEG-PDLLA nanoparticles

Temperature-dependant solubility of G3.0-PEG-PDLLA nanoparticles in aqueous phase was confirmed. As the solute becomes more insoluble, the solution will become more turbid and hence one can expect a greater absorbance value. All the nanoparticles were investigated at the same concentration (i.e., 60 wt%) at two temperature points of interest namely, from 25 °C (±1.5ºC) and 37 °C (±1.5ºC). Linear PEG-PDLLA copolymers were used as control. As shown in Figure 16, both linear and dendritic PEG (1500)-PDLLA and PEG (6000)-PDLLA have marginal solubility change with temperature increase. In contrast, linear and dendritic PEG (12000)-PDLLA displays distinctly low solubility at 37ºC.

Figure 16. Solubility test result.



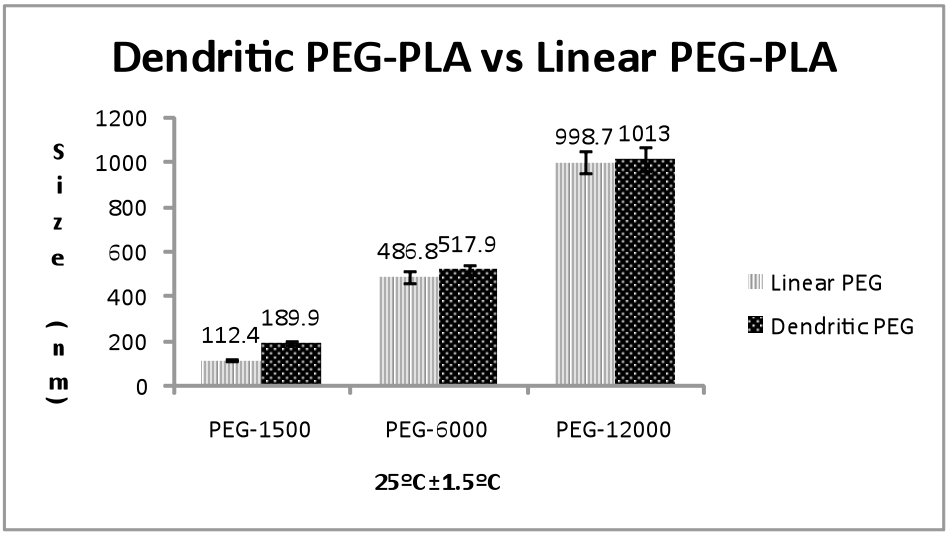
Typically, a temperature-dependent LCST polymer processes a mixture of hydrophilic and hydrophobic blocks. Hydrogen bonding predominates between hydrophilic blocks (i.e., PEG in this case) and water molecules at low temperature, causing improved water solubility; however, temperature increase enhances hydrophobic interactions among the hydrophobic blocks (i.e., PDLLA) and weakens hydrogen bonding interactions, leading to the decrease in the solubility of the polymer. G3.0-PEG-PDLLA of varying PEG length exhibited lower water solubility around 37ºC as compared to their liner polymer counterparts. Further, G3.0-PEG (12000)-PDLLA has lowest solubility around 37ºC as recorded in Figure 16. We reason that hydrophobic interactions among PDLLA chains became strongest around 37ºC and clustering PDLLA on the dendrimer surface

strengthened hydrophobic interactions. We further reason that PDLLA coupled with long PEG 12000 chains obtained more spatial flexibility to interact with other PDLLA segments, causing stronger hydrophobic interactions.

4.4 Self-assembly of dendritic PEG-PDLLA nanoparticles into spherical aggregates with magnified temperature-dependant dimension

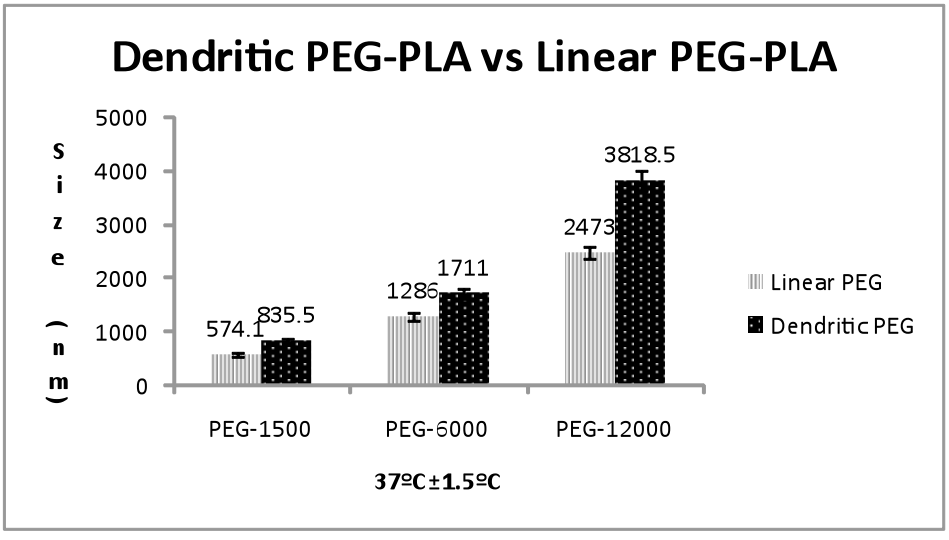
On performing the light scattering test, we observed several results. We compared the characteristics of linear and dendritic PEG-PLA at 25ºC (figure 17). We observed that, there was no significant difference between the particles for the same molecular weight of PEG (t = .26, p-value = 0.753). For example, the size of the nanoparticles of linear vs. dendritic PEG was 998.7±32nm and 1013 ± 48 nm. On performing a student t test, we observed that these sizes are not significantly different from each other [for PEG 1500 (t = .38, p-value = 0.8317) Also, the size of the nano-particles increased with increase in the molecular weight of PEG.

Figure 17. Comparison between dendritic and Linear PEG-PLA at 25ºC



However, at 37ºC, we observed that the size of both linear and Dendritic PEG-PLA increased but, the Dendritic-PEG-PLA increased significantly in size as compared to the linear counterpart (t = .11, p-value = 0.104), as shown in the figure 18.

Figure 18. Comparison between dendritic and Linear PEG-PLA at 37ºC



For PEG-1500, we observed an increase of about 261± 13 nm which was significantly different (Student t-test (t = .13, p-value = 0.072). Similarly we observed an increase of 425±21nm and 1345±87 nm between Dendritic and linear PEG-6000 and 12000 respectively. We found a size increase factor of 3.3 ~ 4.3 fold for particles made of dendritic PEG-PDLLA but the particles made of linear PEG-PLA increased on an average of 2.4~3.1 fold. This confirmed that dendrimer indeed played a role in conferring PEG-PDLLA amplified temperature-sensitivity to expand their size more significantly in response to temperature elevation. More impressively, the size expansion of particles made of dendritic PEG (12000)-PDLLA was twice as much as that of particles made of linear PEG (12000)-PDLLA. From the results obtained, dendritic PEG-PLA can be considered more temperature-sensitive than linear PEG-PDLLA to expand the dimension of particles.

An interesting result that we noted was the capability of PAMAM-PEG-PLA to return back to their original size once the temperature was decreased from 37ºC to 25ºC. This was observed in all the molecular weights of PEG (i.e. 1500, 6000 and 12000). When the temperature was increased back to 37ºC, the size was found to be very similar to the previous 37ºC measurement. This showed us that the addition of the dendrimer makes the change of nanoparticle reversible. This size change was significantly different with linear PEG-PLA (6000 and 12000). The tendency of the dendritic particles to revert back to their original size could not be explained clearly but we believe that this property is a result of the hydrophobic-hydrophilic interactions due to their unique behavior at each of these two temperatures (i.e., room and body). The test results are summarized in Figures 19-24.

Figure 19. Various runs in determining the reversibility of size change for PAMAM-PEG-PLA 1500.

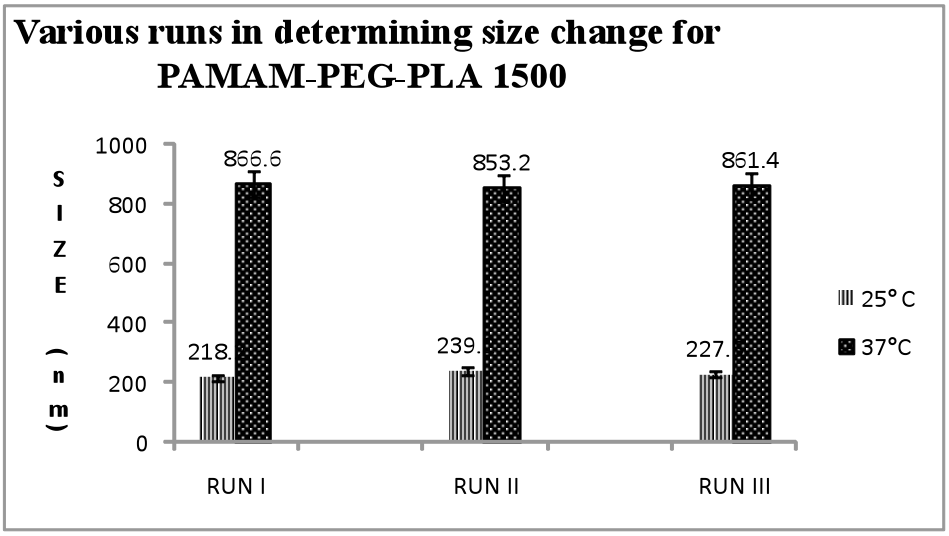


Figure 20. Various runs in determining the reversibility of size change for PEG -PLA 1500.

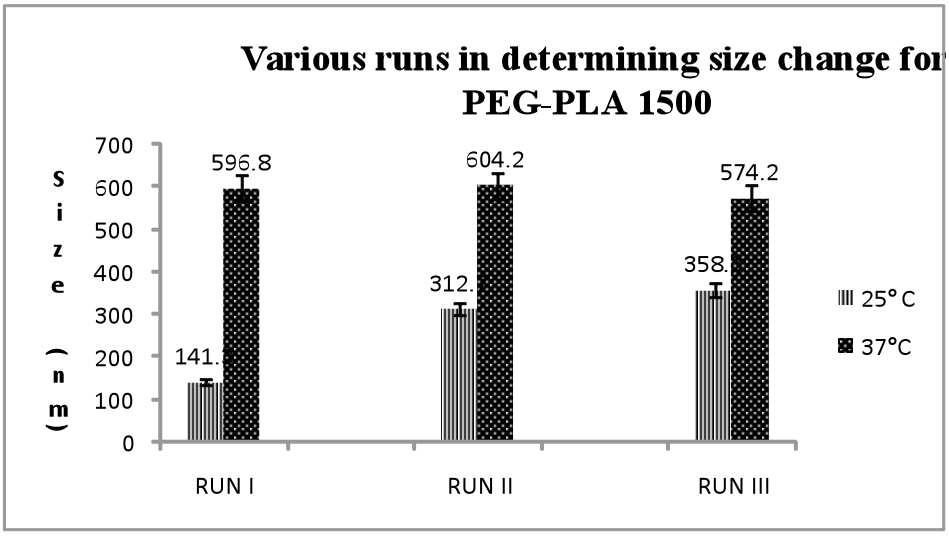


Figure 21. Various runs in determining the reversibility of size change for PAMAM-PEG-PLA 6000.

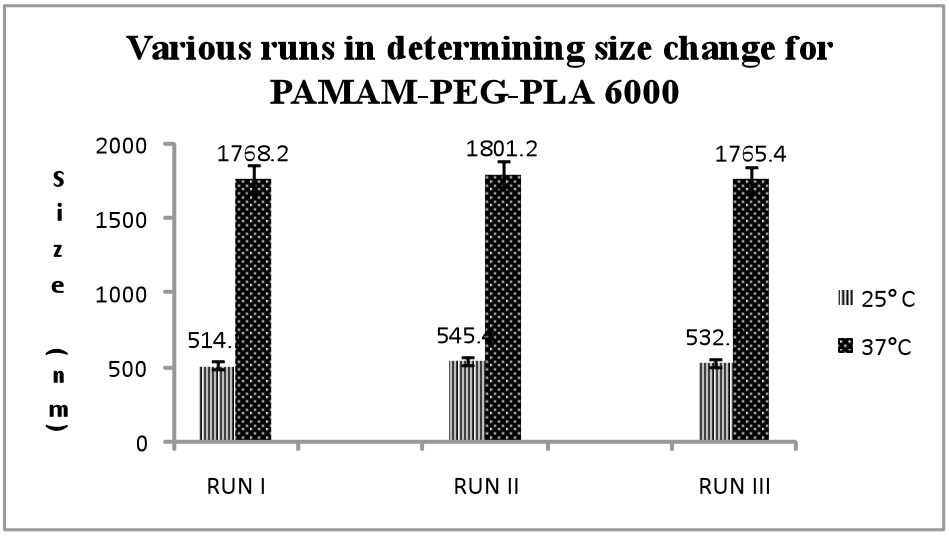


Figure 22. Various runs in determining the reversibility of size change for PEG-PLA 6000.

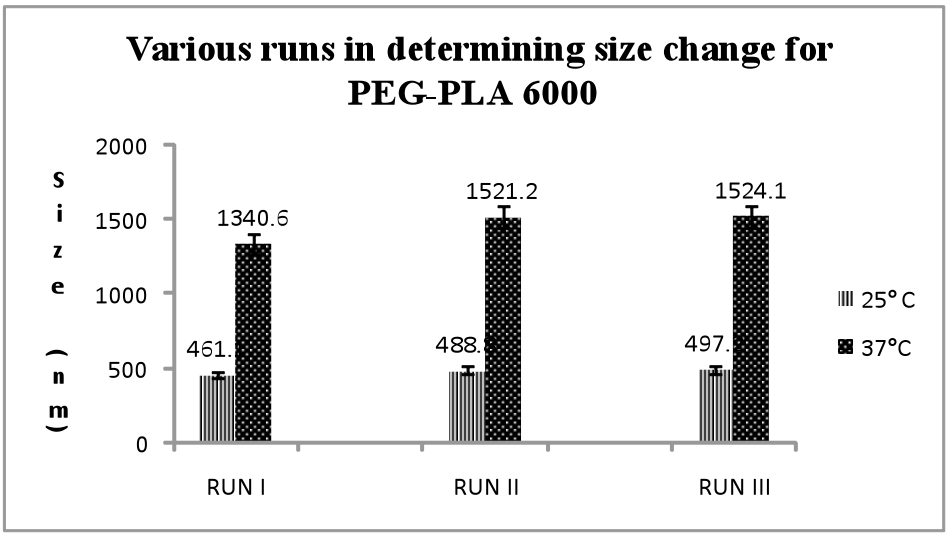


Figure 23. Various runs in determining the reversibility of size change for PAMAM-PEG-PLA 12000.

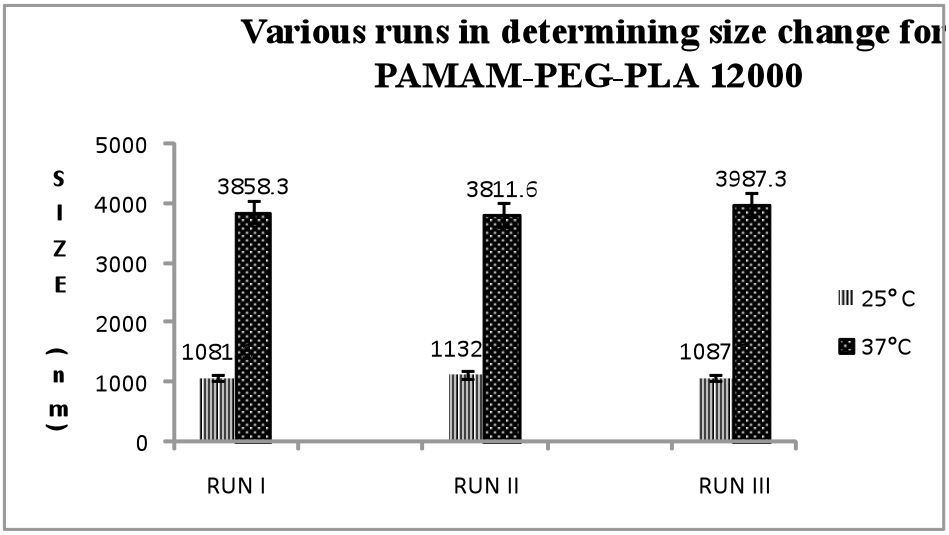
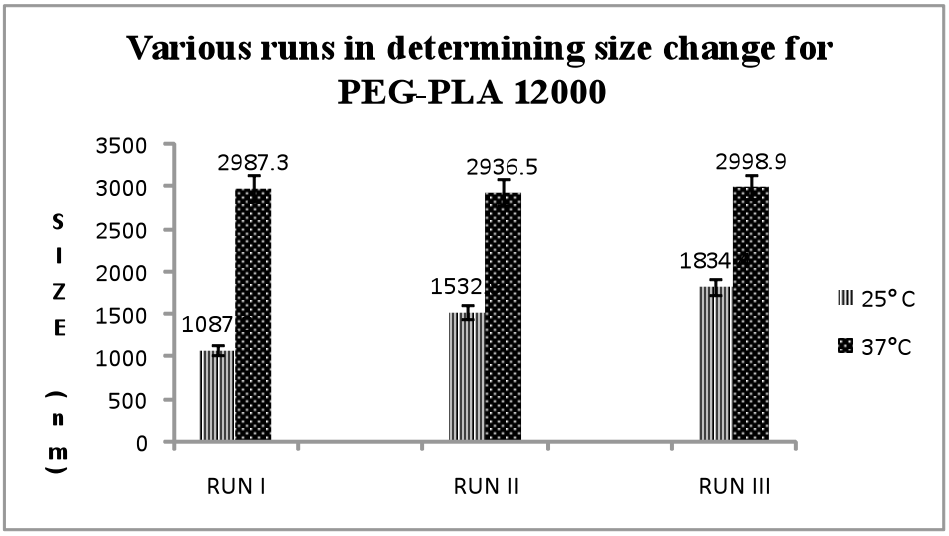
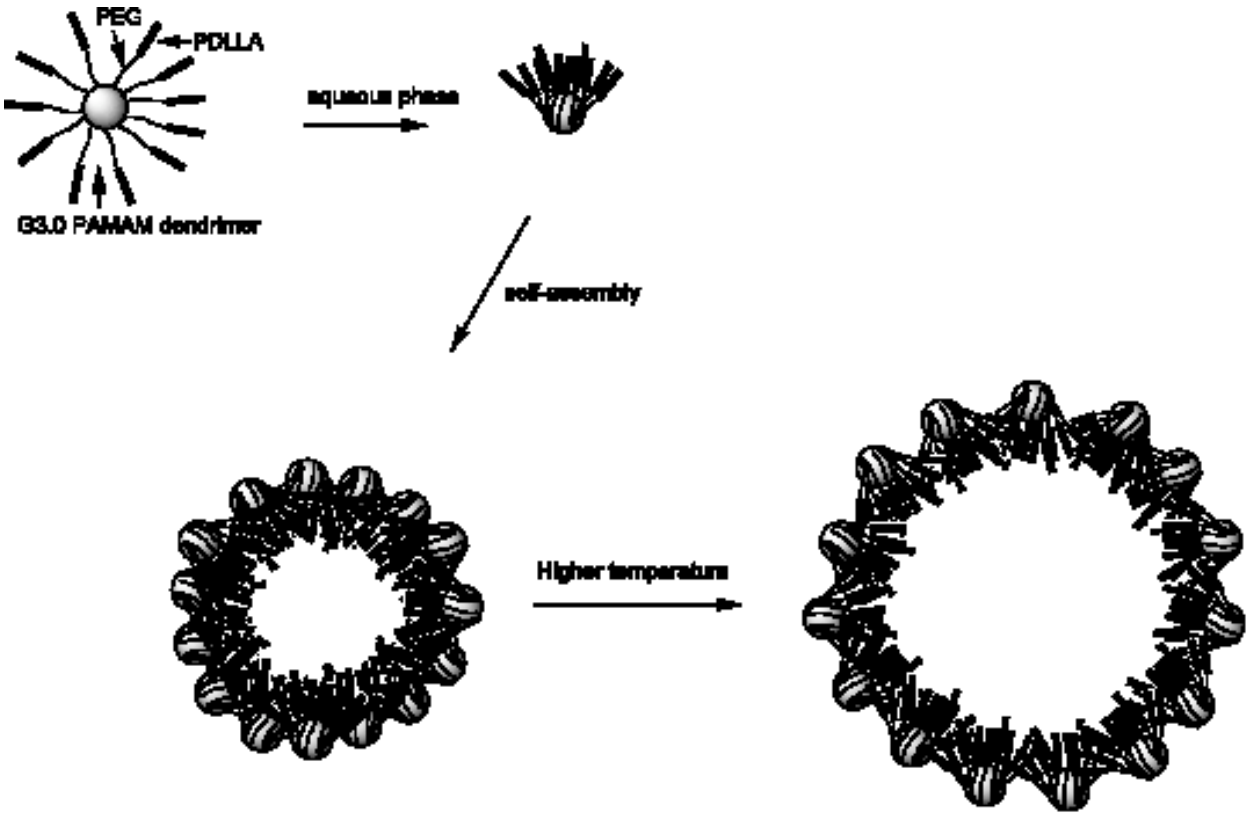


Figure 24. Various runs in determining the reversibility of size change for PEG-PLA 12000.



Based on the above results, dendritic PEG-PDLLA formulated by assembling as many as 23 PEG-PDLLA copolymers on the dendrimer surface were still able to self-assemble into aggregates. Although the exact structure of aggregates made of dendrimer-PEG-PDLLA remains to be elucidated, we believe that highly clustered PDLLA chains yield enhanced hydrophobic interactions at the outer layer. Therefore, we propose that in the aqueous phase, PEG-PDLLA copolymers on the dendrimer surface can reorient their chain structural configuration to make one side of the dendrimer surface more hydrophobic and the other side more hydrophilic, thus leading to the self-assembly of dendrimer-PEG-PDLLA nanoparticles into large spherical aggregates as illustrated in Figure 25. We believe it was the clustering PEG-PDLLA by dendrimer that gave dendritic nanoparticles enhanced amphiphilic surface property and magnified temperature-sensitivity to allow dendritic PEG-PDLLA to self-assemble into spherical aggregates with the capability of expanding their dimension as temperature increases.

Figure 25. Proposed mechanism for self-assembly of dendritic PEG-PDLLA into spherical aggregates with temperature-induced dimension expansion.



Individual G3.0 PAMAM dendrimer has a hydrodynamic diameter of 3.89 nm based on dilute solution viscometry (DSV) measurement[35, 44]. The gyration radius of G3.0 conjugated with 30 PEG (MW=5000 Dalton) chains (i.e., G3.0-PEG (5000)) was reported to be 6.43±0.32 nm based on the Kratky method. G3.0-PEG (6000)-PDLLA synthesized in this study was similar to the reported G3.0-PEG (5000) in terms of PEGylation degree and

PEG length and PDLLA had a low polymerization degree (n=30, Table 4). Therefore, we assumed that individual G3.0-PEG (1500, 6000, and 12000)-PDLLA were similar in magnitude of dimension to the reported G3.0-PEG (5000). From the dynamic light scattering results , we confirmed that all the particles have much larger size than G3.0-PEG (5000) at 25ºC, indicating that dendritic PEG-PDLLA nanoparticles regardless of PEG length could self-assemble into large spherical aggregates. Further, the size of spherical aggregates made of dendritic PEG-PDLLA was found to be highly dependant on the chain length of PEG-PDLLA copolymers: increase in PEG-PDLLA chain length lead to increase in the size of spherical aggregates.

Morphology of spherical aggregates in dehydrated state was also visualized by AFM (Figure 26-28). The average dimension (defined as the maximal length along the elongated direction) of particles made of G3.0-PEG (1500)-PDLLA, G3.0-PEG (6000)-PDLLA, and G3.0-PEG (12000)-PDLLA at 25ºC was 49±23 nm (Figure 26), 64±17 nm (Figure 27), and 213±70 nm (Figure 28), respectively. Spherical aggregates underwent dramatic size reduction as they transitioned from hydrated state to dehydrated state, indicating that they all collapsed on the substrate.

Figure 26. AFM pictures of PAMAM-PEG-PLA (1500) at (a) 25ºC (b) 37ºC

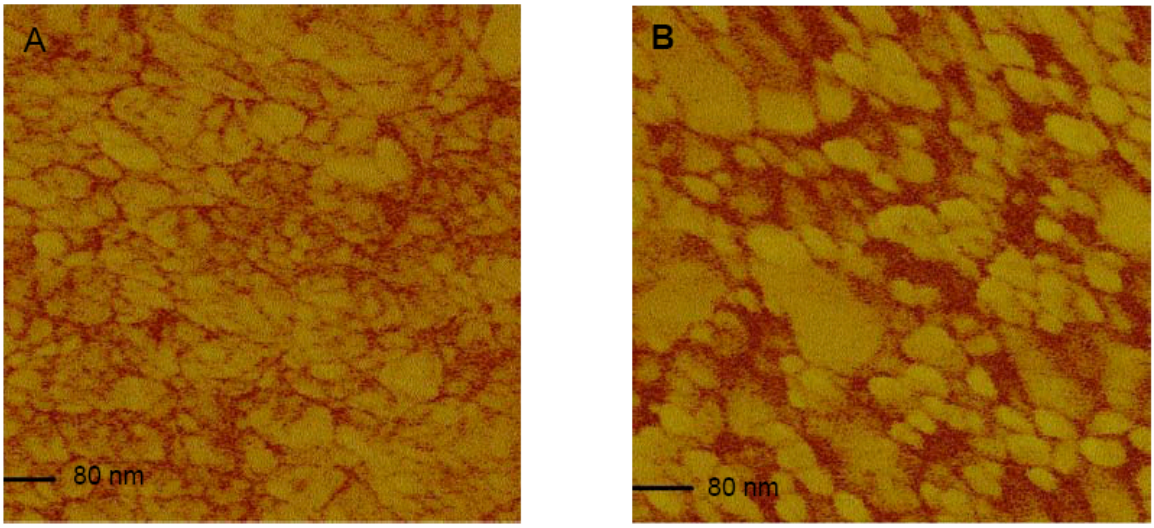
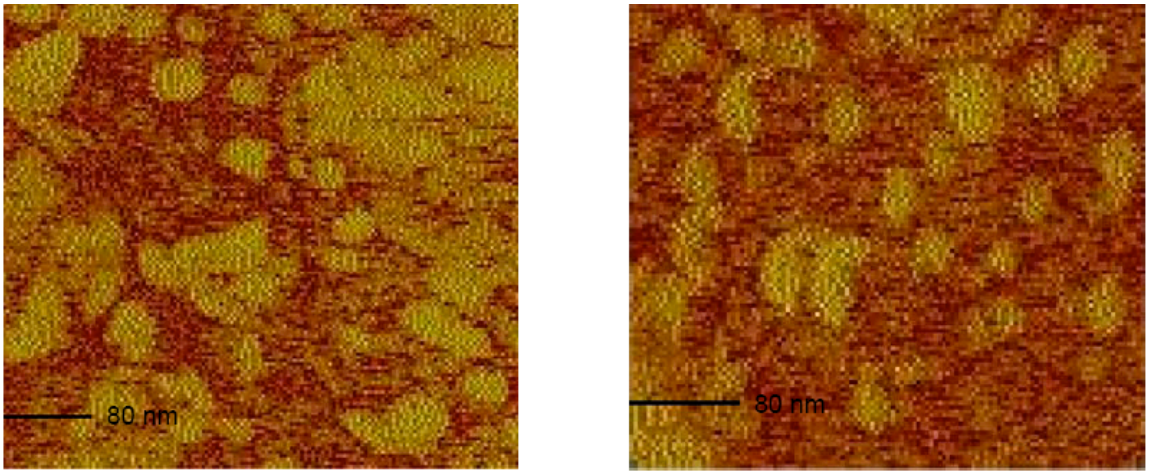


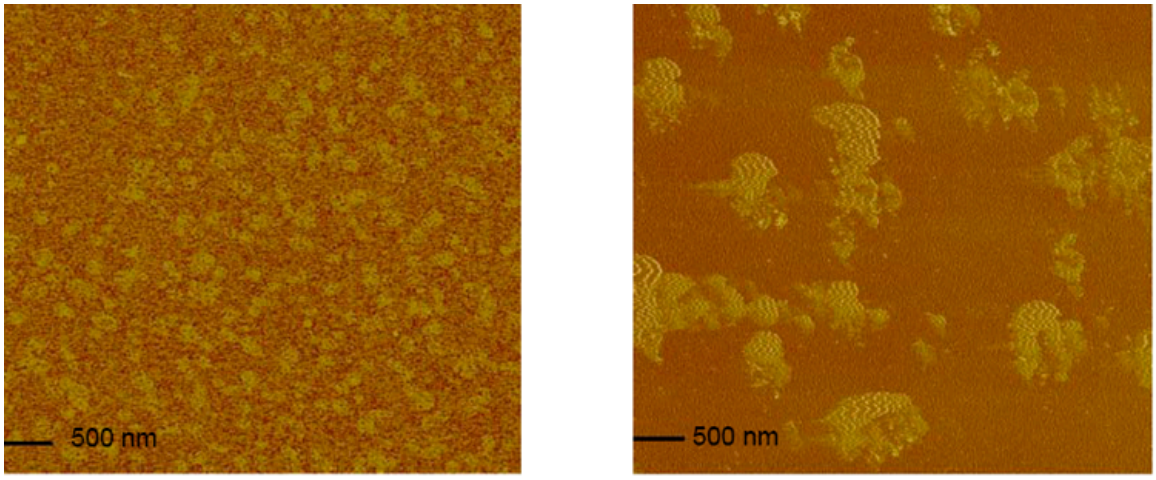
Figure 27.AFM pictures of PAMAM-PEG-PLA (6000) at (a) 25ºC (b) 37ºC



(A) **(B)**

However, the order of the dimension of aggregates in dehydrated state was still consistent with that found in aqueous phase on the basis of PEG-PDLLA chain length on the dendrimer surface. Aggregates made of G3.0-PEG (1500)-PDLLA (Figure 26B) and G3.0-PEG (6000)-PDLLA (Figure 27B) at 37ºC had a dimension of 55±28 nm and 64±17 nm, respectively, which was not significantly different from their dimension at 25ºC according to Student's t-test analysis. (For PEG 6000, t = .34, p-value = 0.841)

Figure 28. AFM pictures of PAMAM-PEG-PLA (12000) at (a) 25ºC (b) 37ºC



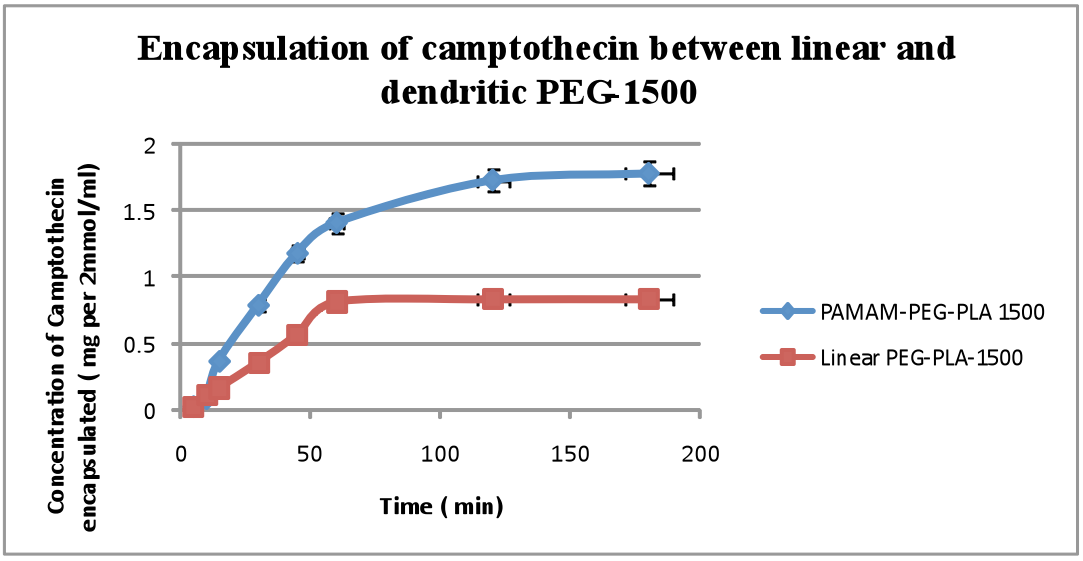
(A) **(B)**

In contrast, the dimension of spherical aggregates made of G3.0-PEG (12000)-PDLLA at 37ºC was 780±200 nm (Figure 28), which was significantly larger than their dimension at 25ºC (t = .13, p-value = 0.152) . Based on AFM images, we further confirmed the self-assembly of dendritic PEG-PDLLA into spherical aggregates and longer polymer chain-based core-shell nanoparticles could formulate spherical aggregates of larger size and potentially higher drug loading capacity.

4.5 Drug encapsulation and controlled release

The saturation concentration of free CPT in water at 25ºC was estimated as 0.91 mg/mL based on our experiment. Drug encapsulation was based on the same molar concentration of PAMAM-PEG-PDLLA (i.e. 2mmol/ml). As shown in Figure 29-31, the maximal amount of encapsulated CPT at 25ºC was raised up to 1.73 mg/mL by G3.0-PEG (1500)-PDLLA and 4.75 mg/mL by G3.0-PEG (6000)-PDLLA, respectively. G3.0-PEG (12000)-PDLLA led to a significant increase in the concentration of CPT to 7.45 mg/mL, i.e., 68% increase when compared to the concentration of CPT in pure water. Encapsulation and release of CPT by linear PEG-PDLLA was investigated for comparison.

Figure 29. Encapsulation of camptothecin between linear and dendritic PEG-1500



We observed that typically particles made by dendritic PEG-PDLLA encapsulated 50% more CPT than particles made of linear PEG-PDLLA copolymers when they have the same molar concentration of PEG-PDLLA. For instance, the dendrimer-PEG (12000)-PDLLA encapsulated approx 7.25 mg of drug whilst its corresponding linear PEG-PDLLA counterpart encapsulated only about 4 mg of drug at 25ºC.

Figure 30. Encapsulation of camptothecin between linear and dendritic PEG-6000

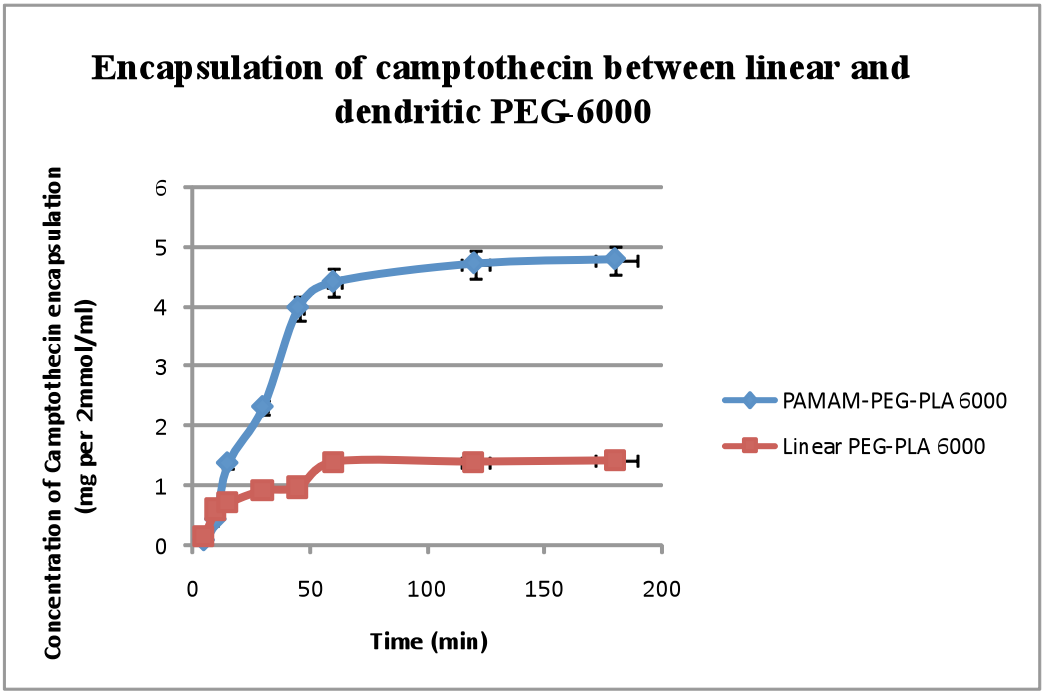
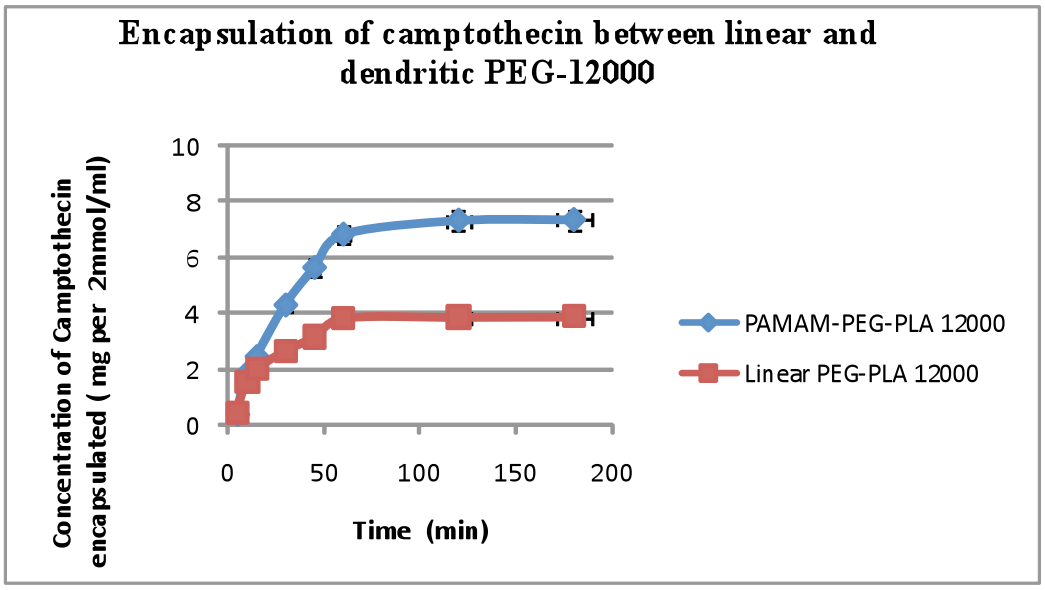


Figure 31. Encapsulation of camptothecin between linear and dendritic PEG-12000



Release studies were performed at 37ºC. We used the CPT-encapsulated dendritic-PEG-PLA and linear PEG-PLA for the study and compared between them. We noticed that the maximally released CPT amount at 37ºC was 1.54 mg/mL by G3.0-PEG (1500)-PDLLA, 2.88 mg/mL by G3.0-PEG (6000)-PDLLA, and 3.42 mg/mL by G3.0-PEG (12000)-PDLLA, respectively (figure 32-34).

Figure 32. Drug release studies of dendritic PEG-PLA vs. linear PEG-PLA 1500

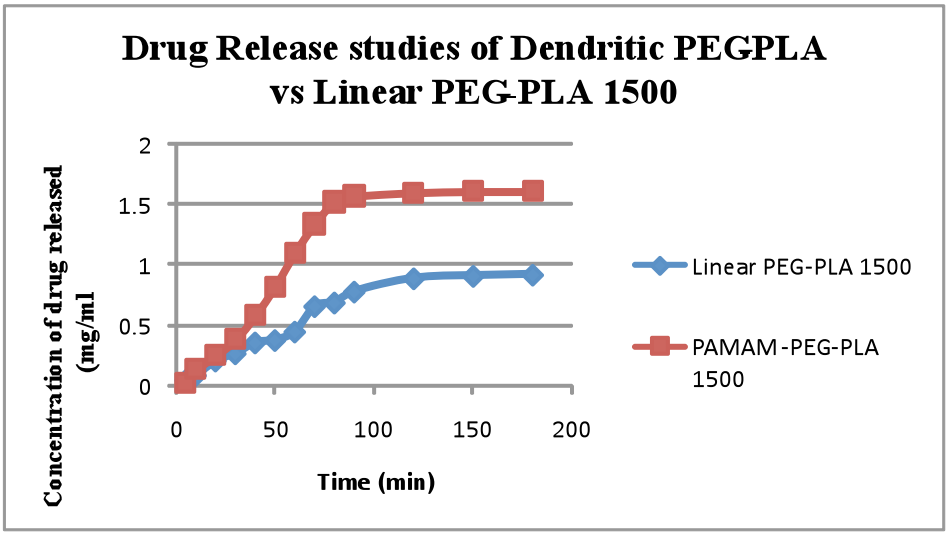


Figure 33. Drug release studies of dendritic PEG-PLA vs. linear PEG-PLA 6000

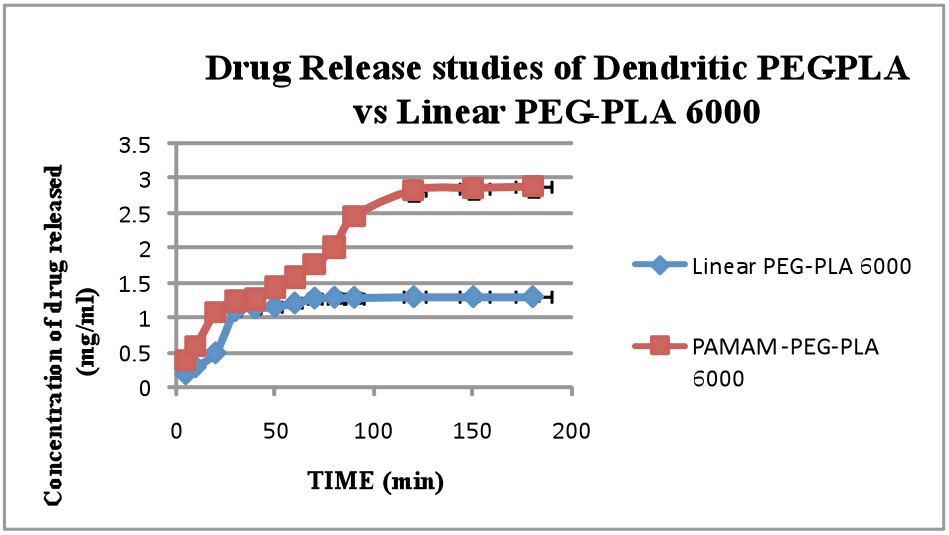
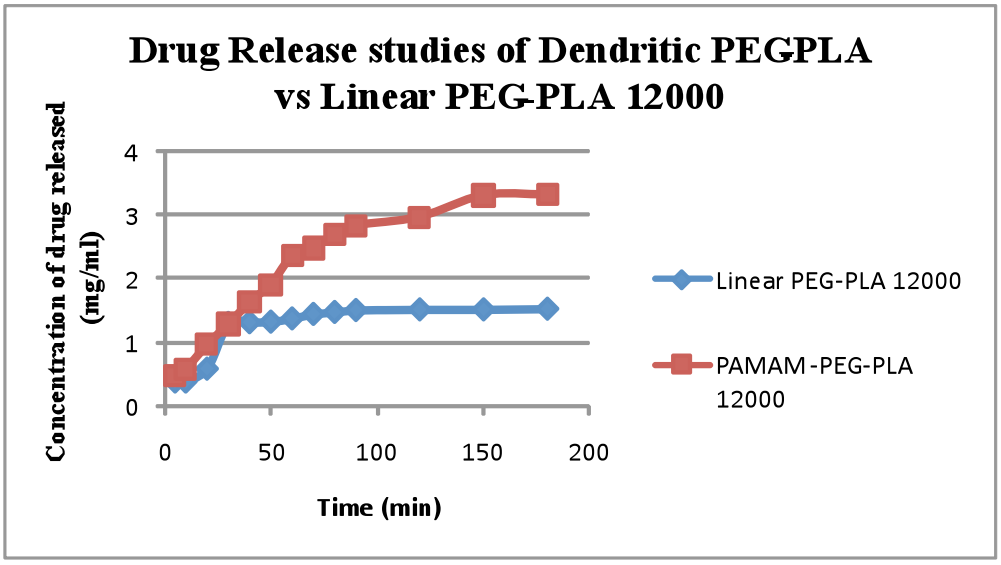


Figure 34. Drug release studies of dendritic PEG-PLA vs. linear PEG-PLA 12000



We also noticed particles made of G3.0-PEG (12000)-PDLLA and G3.0-PEG (6000)-PDLLA had larger encapsulation capacity but quicker release rates than particles made of G3.0-PEG (1500)-PDLLA. The release of Camptothecin within the first 90 min was 2.84 mg/mL·min and 2.45 mg/mL·min for G3.0-PEG (12000)-PDLLA and G3.0-PEG (6000)-PDLLA, respectively, which both were higher than 1.57 mg/mL for G3.0-PEG (1500)-PDLLA.

This study demonstrated that particles assembled by dendrimer-PEG-PDLLA nanoparticles could expand their dimension and hence drug encapsulation capacity in response to temperature increase. Further, drug encapsulation capacity increased as the chain length of PEG-PDLLA assembled on the dendrimer surface increased. Quicker release of CPT from particles made of G3.0-PEG (12000)-PDLLA and G3.0-PEG (6000)-PDLLA than from G3.0-PEG (1500)-PDLLA was probably due to the colloidal instability caused by large molecular weight of dendritic PEG-PDLLA.

4.6 Conclusions

A series of dendritic PEG-PDLLA were synthesized based on PEGylated Starburst™ PAMAM dendrimer G3.0. Dendritic PEG-PDLLA in aqueous phase could self-assemble into spherical aggregates and the dimension of spherical aggregates increased with PEG chain length increase. Further, dendritic PEG-PDLLA exhibited magnified temperature-sensitivity in terms of solubility change and dimension expansion as compared to linear PEG-PDLLA at the same concentration. Our studies also explored the potential use of this new material for drug delivery. Spherical aggregates encapsulate more CPT when dendritic PEG-PDLLA had longer PEG-PDLLA chain and/or when temperature increased to 37ºC. This study demonstrated that assembling PEG-PDLLA long chains on the dendrimer surface magnified their thermo-sensitivity. Successful development of such a new particulate system made of dendritic PEG-PDLLA with an expandable dimension in response to temperature change generated a new direction for designing stimuli-responsive materials.

CHAPTER 5 Summary and Future work

5.1 Summary

Dendrimers are currently being investigated in various applications due to their structure and chemistry. Their unique properties allow them to be novel carriers with the ability to deliver drugs effectively. We investigated the potential use of dendrimers by coupling them to PEG-PLA and studying its characteristics in comparison to that of linear PEG-PLA. We were able to achieve the fact that the addition of dendrimer improved the thermoresponsiveness of the system as a whole and promoted self-assembly. Further, it promoted reversibility of the system, in that the system was able to revert back to its original size when the temperature was decreased. Also, the addition of dendrimer significantly improved the encapsulation and release of Camptothecin drug as compared to its linear counterpart. Hence, we were able to conclude that the structural changes coupled with the hydrophobic-hydrophilic interactions make dendrimers a novel material to be studied for drug delivery.

The PAMAM-PEG-PLA system is highly adaptable and can be engineered for various applications [34, 36]. As the dendrimer network has inner hydrophobic cores included, both hydrophobic and hydrophilic drugs can be loaded. Hence, the new dendrimer based thermo-responsive system developed in this system can be engineered according to the application desired and can hence be used in a wide range of studies.

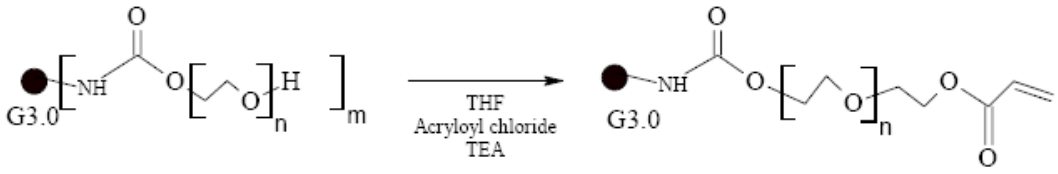
5.2 Future Work

The use of dendrimers in the field of drug delivery is relatively new and hence has ample scope for future studies. We have created a thermoresponsive system and studied drug delivery using Camptothecin, an insoluble anti cancer drug. Further studies can be done using different soluble and insoluble drugs so as to characterize the behavior of this system. Also, mechanical testing can be done to understand the various properties like volume, density, theoretical amount of drug that can be loaded, etc. This will provide us with an in-depth knowledge of how to improvise the system. This study can be progressed by formulating a thermo responsive-controlled drug delivery system to optimize the properties of drug release. The tests done in this study are in vitro. This can be expanded in performing in vivo studies to identify and treat cancer cells. Results of in vivo studies would provide us with a better insight on the drug release kinetics. Further, this project can also be extended to study gene transfection. This study can be taken further to engineer dendrimer based hydrogels. Formulation of such a network can be used in ocular drug delivery and is a potential area to inspect. This study also dealt with synthesizing a dendrimer based hydrogel as explained below.

5.2.1 Synthesis of a PAMAM-PEG-PLA hydrogel

A dendrimer based hydrogel comprising of PAMAM-PEG-PLA conjugated with acrylate was synthesized[40, 45]. As shown in the figure 35, PEG diol was acrylated in order to make photo-initiated crosslinking reaction possible. To convert the free hydroxyl group of PEG on the dendrimer surface to an acrylate group, the reaction procedure involved the following reagents: dendrimer-PEG-OH, acrolyl chloride, and TEA at the respective molar ratio of 1:4:6. G3.0-PEG-OH was dissolved in 5 ml of THF. To this solution a mixture solution of acrolyl chloride and TEA was added dropwise and stirred for 4 hours. Then centrifugation was carried out to remove the salt and the supernatant was collected. The collected supernatant was added dropwise to 40 ml of ethyl ether and kept at -40 ºC for further precipitation. The precipitate was extracted and dialyzed to make sure that excess of acrolyl chloride was removed. The resulting G3.0-PEG-acrylate was then freeze dried.

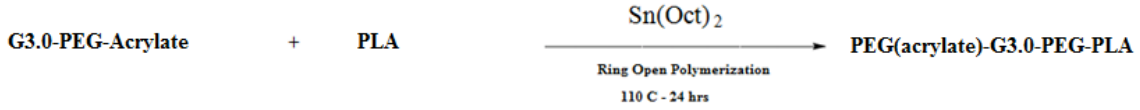
Figure 35. Chemistry for introduction of a UV sensitive double bond to PAMAM-PEG.



The next step involved the conjugation of PAMAM-PEG-Acrylate with lactic acid. Following the similar procedure of conjugation of PAMAM-PEG with LA using ring open

polymerization, the amount of LA to be added to the synthesized PAMAM-PEG-acrylate was calculated. G3.0-PEG-Acrylate was then used as the macromonomer to initiate polymerization of D, L-lactide through the hydroxyl end groups of conjugated PEG chains. The reaction step is summarized in the figure 36.

Figure 36. Ring open polymerization of D, L lactide with G3.0-PEG-acrylate.



To study the distribution of dendrimer within the hydrogel network and observe cell internalization PAMAM dendrimer, G3.0 was labeled with florescent dye, i.e. FITC. G3.0-PEG-acrylate was dissolved in 2 ml of PBS buffer, and FITC was dissolved in 1 ml of methanol. The FITC solution was added dropwise to PBS solution of G3.0-PEG acrylate in which the molar ratio of amine groups of dendrimer to FITC was 1:1.25. This mixture solution was then stirred in dark for 24 hrs. Dialysis was then performed to remove the excess amount of FITC. The solution was then freeze dried to obtain FITClabeled G3.0-PEG-acrylate.

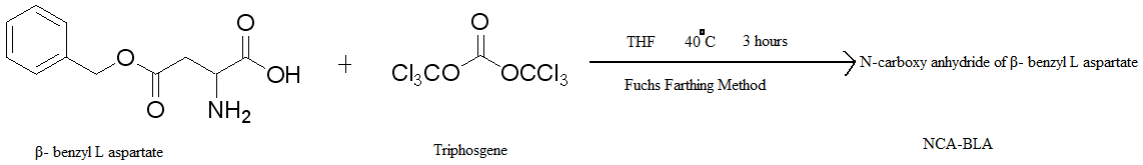
Having synthesized this hydrogel, a preliminary water swelling test was performed. However, this test was inconclusive because the hydrogel created was very unstable and

degraded very quickly. Further studies can be done in this area to improvise the characteristics of the hydrogel and perform other tests.

5.3.2 Synthesis of a pH-Sensitive Polymer.

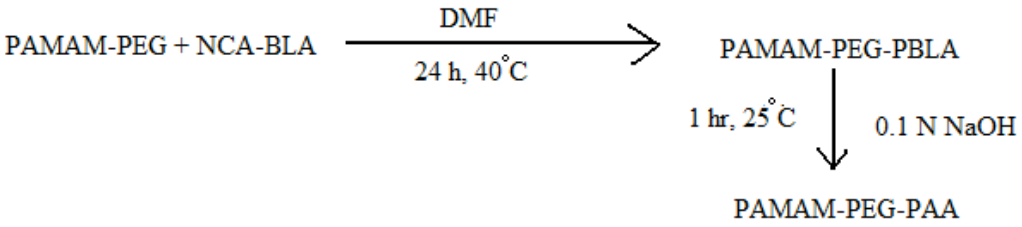
Our primary aim was to create a temperature-sensitive drug delivery system; one kind of stimulus that can be used in drug delivery. Ongoing studies are being done in creating a novel pH-responsive material and studying the characteristics, similar to what is done in this study. A novel pH-responsive material based on PAMAM-PEG-PAA was synthesized and characterized using NMR. The synthesis comprised of two steps. The first step was to synthesize N-carboxy anhydride of β- benzyl L aspartate (NCA-BLA) by Fuchs Farthing method. β- benzyl L aspartate (5 g, 30.3 mmol) was suspended in 50 ml of THF and heated to 40 °C in a nitrogen atmosphere. A solution of 3 g (12.1 mmol) of triphosgene dissolved in THF was added dropwise to the stirred reaction mixture. After 3 h, the reaction mixture was filtered to remove any insoluble materials and the filtrate was poured into 300 ml of hexane. The resulting suspension was stored at −20 °C overnight to assure complete crystallization. For further purification, the obtained NCA-BLA was recrystallized three times from a mixture of THF/*n*-hexane and dried at 25ºC in a vacuum.

Figure 37. Synthesis of NCA-BLA by Fuchs-Farthing method



The second step involved conjugation of PAMAM-PEG (as synthesized previously) to PAA. PAMAM-PEG (1.0 g) was dissolved in 10 ml of DMF. Then a solution of NCA-BLA (0.35 g) in 4 ml of DMF was added to the solution of PEG. The reaction mixture was stirred for 24 h at 40 °C and then precipitated with an excess of diethyl ether. The precipitate was dissolved in 10 ml of chloroform and then reprecipitated into an excess of diethyl ether. The benzene groups were removed by placing the PEG-PBLA in 0.1 N sodium hydroxide at 25ºC for about 1 hour. PAMAM-PEG-PAA was hence prepared. The summary step is shown in figure 38.

Figure 38. Synthesis of PAMAM-PEG-PAA



Similar to this study, a control was also synthesized comprising of PEG-PAA. Figure 39 and fig 40 shows the confirmation of synthesis of PAMAM-PEG-PAA 12000 and PEG-PAA –12000 by ^{1}HNMR. The peaks from 2ppm to 3ppm are comparable to our synthesis of PAMAM-PEG-PLA (fig 10 and 12) and denote the peak for the PAMAM dendrimer. Similarly the PEG peaks (3ppm to 4 ppm) are identical to that obtained in the previous synthesis. The peaks obtained from 0 to 1 ppm belong to PAA since we obtained the similar pattern in Fig 39 and 40. This confirmed the synthesis of PAMAM-PEG-PAA and further tests have to be done in order to determine its functionality as a pH- sensitive polymer.

Figure 39. ^{1}H-NMR of PAMAM-PEG-PAA (12000)

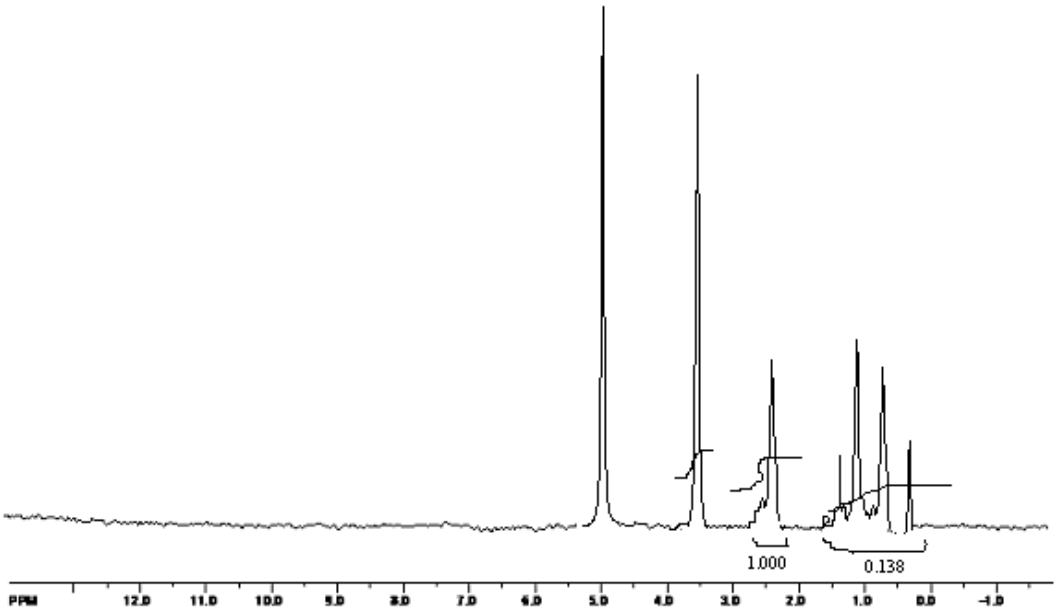
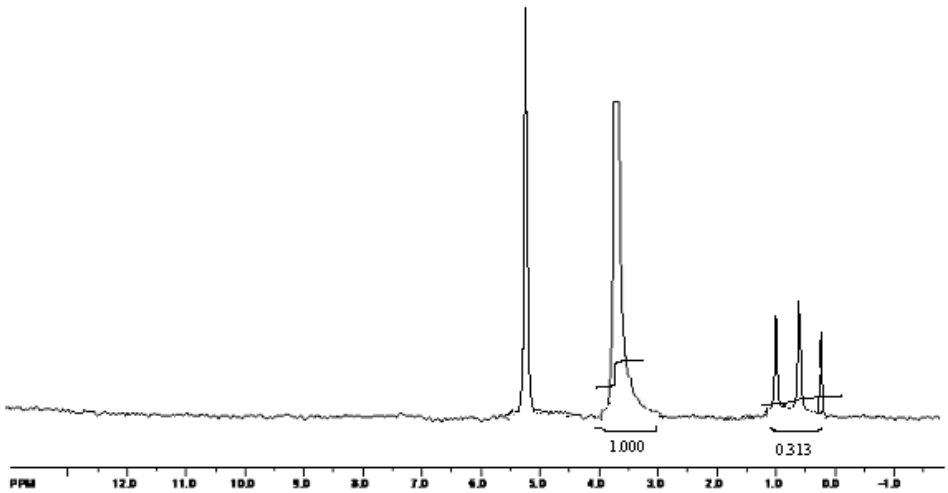


Figure 40. ^{1}H-NMR of PEG-PAA (12000)



Having synthesized this material, further tests are being carried out, in a similar manner as performed for PAMAM-PEG-PLA. The results of these two studies can be compared and this would pave the way for creation of an optimum drug delivery system.

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Literature Cited

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