2008

Synthesis and Characterization of Polyionic Hydrogels

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SYNTHESIS AND CHARACTERIZATION OF POLYIONIC HYDROGELS

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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August 2008

Acknowledgement

I would like to thank my research advisor, Dr. Hu Yang for his patience, guidance and support over the course of this research. He was a constant inspiration, and his assistance and suggestions were invaluable towards the completion of this work.

I would also like to thank the members of my thesis committee, Dr. Gary Bowlin and Dr Thomas Haas, for their efforts in reviewing and evaluating my research. I would like to acknowledge and thank the Department of Biomedical Engineering at Virginia Commonwealth University for the various opportunities that have helped to make this journey an educational as well as an enjoyable one. I would also like to thank Alicia Freshwater-Smith, Arunvel Kailasan, and Quan Yuan for their helpful insights and invaluable suggestions throughout the course of this research.

I would like to dedicate this research work to my family. To my husband who has been my major inspiration, guide and support to accomplish my degree. I would also like to thank my parents, who have always encouraged me to pursue my goals, work harder to succeed and win over defeat. From them I have also learned to do my level best. Finally but not the least I would like to thank my sister, who is my best friend. Thank you to all for being a part of my life.

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Abstract

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By Pooja N Desai M.S.

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In this study we describe novel polyionic dendrimer – PEG hydrogels for drug delivery. Hydrogels have a crosslinked insoluble network of polymer chains, which have found many applications including drug delivery and tissue regeneration. Dendrimers provide an ideal platform for drug delivery as they possess a well-defined highly branched nanoscale architecture with many reactive surface groups. Their highly clustered surface groups allow for targeted drug delivery and high drug payload to enhance therapeutic effectiveness. This study presented a new type of polyionic hydrogels based on dendrimers with potential applications in drug delivery and tissue engineering.

Polyethylene glycol (PEG) with various chain lengths [1500, 6000 , 12000 Da] was first conjugated to the Starburst™ G3.0 PAMAM dendrimer to form stealth dendrimers through one ending site of PEG using p-nitro phenyl chloroformate and Triethylamine. The free hydroxyl group of PEG was further converted to an acrylate group using acrolyl chloride and Triethylamine. The conjugation was characterized with ^{1}H-NMR. The Ninhydrin assay was used to estimate the loading degree of PEG on the dendrimer surface. The molecular weight and loading degree of PEG was varied. Hydrogel formation was realized by subjecting dendrimer-PEG acrylate to UV exposure for a brief period of time at the presence of Eosin Y, Triethanolamine [TEOA] and 1 vinyl 2 Pyrrolidinone [NVP] photo initiator system. Viscosity increase was observed after hydrogel formation. PEGylated G3.0 PAMAM dendrimer served as cross-linking agent to form hydrogels because of its multiple functionalities. PEGylated half generation dendrimer G3.5 was subjected to hydrogel formation and its swelling behavior was studied. Better hydrogel formation was observed with increased PEG arm length. The surface charges conferred by terminal groups on the dendrimer surface made the hydrogel polyionic with controllable charge density. This new type of hydrogel has many favorable biological properties such as non toxicity and non immunogenecity and multifunctional ties for a variety of *in vivo* applications. Current studies have demonstrated feasibility of chemistry and hydrogel formation. The swelling studies demonstrated pH sensitive behavior. Degradation of hydrogel was observed, for low PEGylated dendrimer degradation also demonstrated pH sensitivity. Controlled drug delivery and release were also investigated. Hydrophobic drug Cyclosprine A was used,

we envision that hydrophobic dendrimer core will used for drug encapsulation and delivery, and later release in controlled fashion. The polymer and hydrogels were evaluated for in vitro cytotoxicity and cell internalization.

CHAPTER 1 Introduction

Diseases have been overpowering mankind for a long time. Researchers have been actively working to develop therapeutic molecules for disease treatment. With new therapeutic agents being developed a major question rises about the effective delivery of these molecules to the target site with minimal side effects. Efforts are being made to develop hydrogels for drug delivery. In general, hydrogels have a polymer network, which is able to absorb and retain water, encapsulate therapeutic molecules and later allow sustained and controlled drug release. Various natural and synthetic materials have been used to form hydrogels. Hydrogels can be classified into ionic, non ionic, and neutral hydrogels. Ionic hydrogels have the ability to respond to the change of pH, hence termed as pH sensitive hydrogel. Ionic hydrogels have two main structure features: a penetrable network and a number of fixed charges. The penetrable network allows the exchange of solute and water. Fixed charges are responsible for the regulation of the electrochemical balance between the hydrogel and the surrounding medium. The swelling of ionic hydrogels is governed by pH [1]. For instance, a hydrogel network containing acidic groups swells at high pHs but shrinks at low pHs [2]. Therefore ionic hydrogels have been used for delivery of various therapeutics and controlled drug release based on pH adjustment [3, 4].

The objective of this study was to develop a novel polyionic hydrogel based on polyamidoamine (PAMAM) dendrimers. PAMAM dendrimers have demonstrated great promise for a variety of biomedical applications because of their many attractive features including well-defined nanoscale chemical structure, globular shape, low polydispersity (close to 1.0), biocompatibility, and a number of reactive surface groups [5]. Dendrimer has a highly branched nanoscale structure, which falls into the range of the sizes of biological components within the body. PAMAM dendrimers possess a number of charged surface groups (i.e., amine or carboxylate), which can confer pH sensitivity and can be used for forming ionic hydrogel with proper modification such as crosslinking. In addition, the core of dendrimer is capable of encapsulating hydrophobic compounds. Compared with linear polymers, their many accessible reactive end groups at the surface are more suitable to deliver agents of interest and achieve a high payload within a compact scaffold [6, 7]. Developing a dendrimer-base polyionic hydrogel network will integrate the structural advantages of both dendrimer and hydrogel. In this study PEG was used as a spacer to link individual dendrimer molecules. Covalent attachment of PEG to the dendrimer was on the basis of its many useful properties such as biocompatibility, stealth properties, prevention of protein absorption, etc. The project aims to: 1) explore optimal hydrogel formation conditions; 2) determine the effect of polymer structure on gel formation; and 3) evaluate its pH sensitivity, degradation and cytotoxicity as well as drug release.

CHAPTER 2 Background

Polymeric drug delivery systems have been extensively studied in order to solve the potential problems associated with drugs or bioactive molecules including toxicity, site dependence, low effectiveness, poor solubility, short half life, rapid degeneration and rapid clearance form the body, etc [8]. Polymeric drug delivery systems can help:

1. Reduce toxic effects on the healthy tissue and reach sites that are conventionally inaccessible due to the presence of various barriers [9] by targeted drug delivery;
2. Increase the half-life of drugs, preventing their rapid degradation, and reduce the rate of elimination, thus maintaining drug concentration within a therapeutically effective window;
3. Reduce the amount of drug required to achieve therapeutic efficacy;
4. Cut down the number of repeated invasive dosage required for certain conditions and thus helps to improve patient's compliance and offers better living.

Considering various properties such as flexibility, structure, biocompatibility, and hydrophilicity, three dimensional matrices, *hydrogels*, are being extensively used as drug delivery carriers [10].

2.1 Classification of hydrogels

Hydrogels can be classified based on different criteria.

Table 1. Classification of hydrogels[10]

Classification	Types
Source	Natural
	Synthetic
Component	Homopolymer (one type of hydrophilic polymer)
	Copolymer (two types of plolymers, at least one hydrophilic
	Multipolymer (more than three types of polymers)
	Interpenetrating polymer network
Electric or Ionic charge	Neutral hydrogels
	Anionic hydrogels
	Cationic hydrogels
	Ampholytic hydrogel
Structure	Physical structure
	Amorphous hydrogels (chains randomly arranged)
	Semicrystalline hydrogels (dense regions of ordered macromolecules, i.e. crystallites)[10]
	Hydrogen-bonded hydrogels
	Crosslinked
	Covalent bond
	Intermolecular force
Functions	Biodegradable
	Stimuli responsive [11]
	pH, light, temperature, etc

Table 2. Commonly used natural and synthetic polymers for hydrogel preparation [10]

Type	Example
Natural	Chitosin
	Alginate
	Fibrin
	Gelatin
	Collagen
	Dextran
	Hyaluronic acid Acrylic acid (AA)
Synthetic	Hydroxyethyl methacrylate (HEMA)
	N-(2-hydroxypropyl) methacrylate (HPMA)
	N-vinyl-2-pyrrolidone (NVP)
	N-isopropyl acrylamide (NIPAAm)
	Vinyl acetate (VAc)
	Methacrylic acid (MAA)
	Polyethylene glycol acrylate/methacrylate (PEGA/PEGMA)

2.1.1 Homopolymeric and copolymeric hydrogels

Homopolymer is composed of a number of identical monomer units. Homopolymeric hydrogels may have a crosslinked or uncrosslinked network. Crosslinked homopolymeric hydrogels can be used to develop sustained and controlled drug delivery devices, contact lenses, etc. Some of the crosslinked homopolymers that have been used are poly (hydroxyalkyl methacrylate) derivatives include poly (3-hydroxypropyl methacrylate (PHPMA), poly (glyceryl methacrylate) (PGMA) and poly (2-hydroxyethyl methacrylate) (PHEMA) [12]. Uncrosslinked homopolymers have also been used for forming hydrogels, such as poly (N-vinyl-2-pyrrolidinone) (PNVP) [13],

poly(acrylamide) (PAM), poly(ethylene glycol) (PEG) and poly(vinyl alcohol) (PVA). However, uncrosslinked hydrogels have poor network stability. Copolymeric hydrogel networks are comprised of two or more different monomers, which are arranged in block or alternating configuration along the chain of the polymer. One of copolymeric hydrogel examples is based on N-vinyl-2-pyrrolidone (NVP) and polyethylene glycol diacrylate (PAC) [14].

2.1.2 Interpenetrating polymer networks

Typically interpenetrating polymer networks (IPNs) combine two polymers in a single hydrogel network. To be considered as IPN, three conditions should be satisfied: (1) the two polymers are synthesized and/ or crosslinked in the presence of the other, (2) the two polymers have similar kinetics, and (3) the two polymers are not dramatically phase separated [15]. As the two polymer components incorporated in IPN are not covalently bound but at the same time inseparable form the network, IPN can retain characteristics of both polymers [2]. One or both macromolecules of the IPN could be biodegradable [16].

2.1.3 Ionic or nonionic hydrogels

Ionic hydrogels, also known as polyelectrolytes, can be prepared from monomer possessing ionic charges. The charges can either be positive or negative; therefore, the hydrogels can either be cationic or anionic, respectively. These hydrogels can respond to pH change and hence are also termed as pH sensitive hydrogels. Hydrogels can even possess both negative and positive charges which are known as polyampholytic hydrogels[17]. The swelling of ionic hydrogels is affected by the ionic interactions between the charged polymer chain and free ions in the medium [18]. For example, ionic hydrogels containing carboxylate groups can absorb a larger amount of water at a pH higher than its pKa because of increased hydrophilicity.

Neutral hydrogels, as the name suggests, do not possess any charged groups. These hydrogels swell to equilibrium when the osmotic pressure of the solvent is balanced with the sub-chain stretching energy. The driving force for swelling is the water-polymer thermodynamic mixing contribution to the overall free energy, and elastic polymer contribution [10]. Ionic hydrogels can help to achieve controlled drug release and may increase the residence time of the hydrogel at the target site. Poly(methacrylic acid-co-methacryloxyethyl glucoside) and poly(methacrylic acid-g-ethylene glycol) hydrogels were reported for insulin delivery, where insulin can be slowly released in acidic medium while at a faster rate in alkaline medium. Ionic hydrogels are also used for self controlled delivery system [19]. Polyacrylic acid (PAA) and chitosan (CS) have been used to formulate hydrogels for sustained release of amoxicillin in the stomach [20].

2.2 Hydrogel preparation

Hydrogel can be prepared by chemically or physically crosslinking polymers. Some commonly used methods are listed in Table 3.

Table 3. Preparation methods for hydrogels [12]

Chemically Crosslinking	Physically crosslinking
Radical polymerization	Ionic interactions
Chemical reaction of complementary groups	Crystallization
	Crystallization in homopolymer systems
Addition reactions	Stereocomplex formation
Condensation reactions	Physically crosslinked hydrogels from amphiphilic block and graft copolymers
High energy radiation	
Enzymes	Crosslinking by hydrogen bonds
	Crosslinking by protein interactions
	Use of genetically engineered proteins
	Crosslinking by antigen–antibody interactions

2.2.1 Chemically crosslinked hydrogels

Chemically crosslinked hydrogels are prepared in the presence of chemical crosslinking agent [12]. Chemically crosslinking imparts structural stability to the network. However, crosslinkers should be non-toxic in order to retain the biocompatibility properties of the gel. There are many methods to realize crosslinking chemically. Crosslinking can be initiated by radical polymerization. The process involved is radical polymerization of low molecular weight monomers in the presence of crosslinking agents, for example hydrogel formation via the polymerization of HEMA in the presence of a suitable

crosslinking agent (e.g. ethylene glycol dimethacrylate). Polymer chains can be crosslinked to form a network by chemical reaction of complementary groups, through which functional groups and complementary reactive groups react to form covalent bond, such as amine-carboxylic acid or isocyanate-OH/NH_2 reaction, or Schiff base formation. Polymers with hydroxyl groups (e.g. poly (vinyl alcohol) (PVA)) can be crosslinked by using glutaraldehyde, amine containing polymers can be crosslinked by Schiff base [21].

Hydrogel can also be formed from the water soluble polymers with higher functional crosslinking agent using addition reaction. For example polysaccharides can be crosslinked with divinylsulfone [22]. The properties of the hydrogel network can be controlled by varying the concentration of the dissolved polymer and the amount of crosslinking agent. In the method of crosslinking by condensation reaction, the commonly used crosslinking agent to crosslink water soluble polymers with amide bonds is *N*, *N*-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide (EDC) [23].

Alternatively gamma radiation and electron beam methods are used to form hydrogels. For example, hydrogel can be formed by subjecting water soluble polymers derivatized with vinyl groups to high energy radiation [24]. Hydrogels are also formed from polymers having acrylate end groups by using a suitable photoinitiator [25]. Polyvinyl alcohol and PEG can be modified to have acrylate groups for hydrogel formation based on this method [26]. In addition the use of enzymes as crosslinking agents has been explored. For example, transglutaminase was applied to crosslink functionalized PEG and a lysine-containing polypeptide [27].

2.2.2 Physically crosslinked hydrogels

A major drawback of chemically crosslinked hydrogels is that the presence of crosslinking agents may cause toxic effects and raise hindrance for loading of therapeutic agents within the matrix [12]. Physically crosslinking utilizes noncovalent interactions between polymer chains to form hydrogels. This helps to eliminate the toxic effects imparted by chemical crosslinking agents. Ionic interactions can cause hydrogel formation, such as alginate [28]. Chitosan-based hydrogels have been formed with glycerol-phosphate disodium salt [29]. The crystallization method including alternate freezing and thawing is also one of the ways of forming hydrogels. One example is water soluble poly(vinyl alcohol) PVA. It can form a highly elastic hydrogel [30]. Stereocomplex formation between enantiomeric oligomeric lactic acid chains can form hydrogel. Polylactic acid (PLA) is one such polymer which can form stereocomplex, first reported by Ikada et al [31]. Examples include PLLA–PEG–PLLA and PDLA–PEG–PDLA stereocomplex [32], pHEMA (poly(HEMA-*g*-oligolactate)s) etc. Hydrogel can also be formed after mixing aqueous solutions of dex-(l)-lactate (l-lactic acid oligomer grafted to dextran) and dex-(d)-lactate [33]. Some types of polymers such as amphiphilic diblock copolymers, multiblock copolymers or graft copolymers can self assemble into an organized structure to form hydrogels [12, 34]. Hydrogels can also be formed via hydrogen bonding [35, 37]. Crosslinking by protein interactions is another way to prepare physically crosslinked hydrogels. In particular genetic engineering has been applied for preparation of hydrogels based on protein interactions. DNA determines protein

synthesis. Therefore its physical and chemical properties can be precisely controlled by changing synthetic DNA sequences with genetic engineering tools [12, 38]. Proteins exhibit coiled structures formed from helices. Conjugation of proteins to synthetic polymers has been used to take advantage of the properties of both proteins and synthetic polymers. Hybrid hydrogels can be formed on the basis of polymer poly (*N*-(2-hydroxypropyl)methacrylamide) (PHPMA) and two types of coiled proteins: natural (motor protein kinesin) and synthetic (HHHHHH-(randomly structured tail)-$(VSSLESK)_6$) [39]. Antigen-antibody reaction has also been used to form interpenetrating polymer network by immobilizing both antigen and antibody to polymer chain. For example, rabbit immunoglobulin G (rabbit IgG) and goat anti-rabbit IgG (GAR IgG) as the antigen and antibody for hydrogel formation have been explored [40].

2.3 Properties of hydrogels

Synthetic hydrogels are crosslinked insoluble polymer networks that swell in aqueous solutions and have been widely used because of their biologically favorable properties such as biocompatibility, high capacity for water absorption similar to hydrodynamic properties of hydrogels formed from natural material and to that of biological tissues [18], as well as their minimal mechanical irritation due to their soft and rubbery state [41]. Hydrogels have found applications in controlled drug delivery and release, gene delivery, wound healing and tissue scaffolding [42, 43]. The properties of hydrogels can be

modulated through appropriate engineering of polymer structures that form the network of the hydrogel.

In particular, hydrogel based drug delivery systems should meet the requirements of certain pharmacological parameters including bioavailability, biodistribution and pharmacokinetics. Bioavailability is defined as the measure of therapeutically active drug that reaches the systemic circulation and is available at the site of action. These systems thus help to increase the half life of the administered drugs and improve their bioavailability. The way, in which the targeted biomolecule's path of travel within the experimental subject is tracked, is termed as biodistribution. The deposition of the drug in various tissues can be modified by targeting a specific tissue or cell type of interest based on hydrogel drug delivery systems. Nowadays challenges exist in developing drug delivery systems for avoidance of toxicity, controlled biodegradation and subsequent elimination from the body, and cost reduction for manufacturing. Hydrogels should be nontoxic and preferably biodegradable. The toxicity of hydrogels can arise due to un-reacted monomers, oligomers or photo-initiators. In order to create biocompatible hydrogels, repeated washing is necessary to eliminate contaminants from hydrogels. Also, kinetics of polymerization should be thoroughly studied so as to achieve enhanced conversion rates, hence minimizing un-reacted monomers and side product.

In most cases the mechanical strength of hydrogels is not of major importance, as for drug delivery the network eventually breaks down. But for applications in which the gel matrix needs to maintain the mechanical integrity such as adhesive sealants for wound

closure, hydrogel should possess a certain degree of mechanical stability. Mechanical stability of the hydrogel is also needed when the hydrogel is required to withstand environmental conditions within the body such as extreme pH, before it reaches the target site. Meanwhile, it should be mechanically flexible enough to facilitate release of incorporated therapeutic agents. Thus a balance between mechanical strength and flexibility is essential. For instance, tissue adhesive hydrogels should be mechanically stable to resist forces within the body such as intraocular pressure when the gels are used to seal the ocular wounds, or external forces such as pressure and shear force for wound healing applications.

2.4 Hydrogels for drug delivery

Hydrogels now play an important role in drug delivery. Hydrogels as cell adhesion resistant surface have been used to prevent thrombosis after surgery. Hydrogels have compatibility with blood and other body fluids, thus their application can be extended for forming contact lenses, wound dressings, membranes, and as coating applied to living tissue surfaces. Hydrogel can encapsulate cells and allow them to proliferate, differentiate, and organize, indicating their potential in tissue engineering scaffolding. Drug delivery has been a major application for hydrogels. Hydrogels of various functionalities can be developed to meet different biomedical requirements. Stimuli-sensitive hydrogels can respond to changes in various parameters such as pH and

temperature to achieve controlled drug release. For instance the digestive tract has a pH range from 4-8. Controlled drug release can be realized in the digestive tract by designing a pH sensitive hydrogel. Hydrogel based delivery can be used for epidermal and subcutaneous application. If hydrogels exhibit tissue adhesiveness they can be used towards applications such as closure wound healing for various tissues or organs. In addition this adhesive property can be used for drug delivery through mucus membranes to achieve non-invasive drug administration. Hydrogels having "stealth" properties due to presence of PEG have extended in vivo circulation by preventing the activation of the host immune response such as, phagocytic activity [44].

2.4.1 Oral drug delivery

Oral administration of drug has been practiced for a long time because of the ease of its application. Hydrogels have been used for maximizing the absorption of the drug administered. pH-Sensitive chitosan– polyvinylpyrrolidone (PVP) based semi-interpenetrating polymer network have been reported to deliver drugs to treat gastric ailments [45]. Glucose sensitive hydrogels have been reported for effective delivery of glucose. These hydrogels are composed of glucose-containing polymers and PEGylated concanavalin A [46]. Many mucoadhesive hydrogels have found use for mucosal drug delivery as the mucosa has a rich blood supply and is permeable to many drugs. Polymers used for hydrogel formation is based on their adhesive properties including

synthetic polymers such as monomeric cyanoacrylate , polyacrylic acid , hydroxypropyl methylcellulose, and poly methacrylate derivatives as well as naturally occurring polymers such as hyaluronic acid and chitosan. In addition other synthetic polymers such as polyurethanes, epoxy resins, polystyrene, and natural-product cement have also been extensively investigated for the preparation of mucoadhesive hydrogels. Proteins must overcome three natural barriers in the human body: (1) acidic and enzymatic degradation in the stomach; (2) enzymatic degradation in small intestine; and (3) poor intestinal absorption [47]. These hydrogels undergo enzymatic degradation at the desired site. Delivery of drug like beclomethasone dipropionate for the treatment of irritable bowel syndrome has been proposed [48]. The colonic region has also been considered as a possible absorption site for orally administered proteins and peptides due to its low proteolytic activity.

2.4.2 Rectal drug delivery

Hemorrhoids are a commonly occurring rectal disease, which requires an effective drug adsorption at the site rather than a systemic distribution. This need can be addressed by the use of bioadhesive hydrogels that can release drug to the site in a controlled manner and allow for effective removal via biodegradation. Tissue adhesive gels overcome the drawback of slippage of the material *in situ* at the same time non-irritable to the rectal

tissue [49]. The use of xyloglucan polysaccharide gels for rectal drug delivery has been demonstrated [50].

2.4.3 Ocular drug delivery

Eye has various defensive barriers to prevent entry of foreign substances, including effective tear drainage, blinking, and low permeability of the cornea. The existences of these defensive barriers only allow limited drug absorption. Thus repeated doses are needed in order to achieve expected treatment efficacy. To reduce repeated dosing and increase the half life of drugs [51], various approaches have been used such as suspension and ointments, which can be retained in the eye. But their presence causes discomfort to patients and sometimes even leads to adverse reactions. Hydrogels seem to be a suitable carrier for ocular drug delivery because of their physical properties such as elastic and rubbery texture and insolubility in water which resists the ocular drainage system. This system can help achieve better retention and absorption of drugs with the utility of tissue adhesiveness. Further, hydrogels containing drugs can be formed *in situ*. *In situ* hydrogel formation caused by temperature and pressure are also under development and may lead to new ocular drug delivery systems. Hydrogels based on hydroxyethyl methacrylate (HEMA) crosslinked with ethylene glycol dimethacrylate (EGDMA) have been developed for ocular drug delivery [52, 53].

2.4.4 Vaginal drug delivery

Vagina is one of the important sites for drug delivery. *In situ* gel forming polymers that are temperature sensitive have been reported for prolonged and effective release of active drugs such as nonoxynol-9, progestins, estrogens, peptides and proteins[49, 54]. Poloxamers and polycarbophil can also form thermo-sensitive hydrogels for the treatment of fungal diseases especially vaginitis [55].

2.4.5 Transdermal drug delivery

Insulin delivery has been reported through the transdermal route using poloxamer gel [56], which contained a combination of chemical enhancers and iontophoresis to facilitate insulin permeation. This method can also be used for delivery of hormones. Local drug delivery for wound healing is another example of transdermal drug delivery. Hydrogels based on poly(methacrylates) and polyvinylpyrrolidine [57] as well as commercially available hydrogel such as Nu-gel^{TM} have been utilized for transdermal drug delivery.

CHAPTER 3 Experimental methods and material

3.1 Materials

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

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
Starburst™ G3.5 PAMAM dendrimer (20 wt % solution in methanol)	G3.5
Triethylamine	TEA
1-vinyl-2 Pyrrolidinone	NVP
4-nitro phenyl chloroformate	4-NPC
Ethyl ether(anhydrous)	
N,N-dimethylformamide	DMF
Acrolyl chloride	
Ethanol(denatured)	
Ninhydrin	
Eosin Y	
Triethanolamine	TEOA
Dimethoxyphenyl Acetophone	DMPA
Tetrahydrofloron	THF
Deuterium oxide	D_2O
Fluorescein 5(6)-isothio-cynate 90%	FITC
Irgacure 2959	
Hydrochloric Acid	HCl
N,N'-Dicyclohexylcarbodiimide	DCC
Ethanol	
4-Dimethylaminopyridine	DMPA
Phosphate buffer solution	PBS
Cell line RAW264 mouse macrophages	
DMEM medium supplemented with 10%	

fetal calf serum, 100 μ/ml penicillin-streptomycin	
Trypsin solution	
Trypan blue	
Deionized water	DI water
Sodium hydroxide	

3.2 Equipment

Table 4. List of the Equipment used

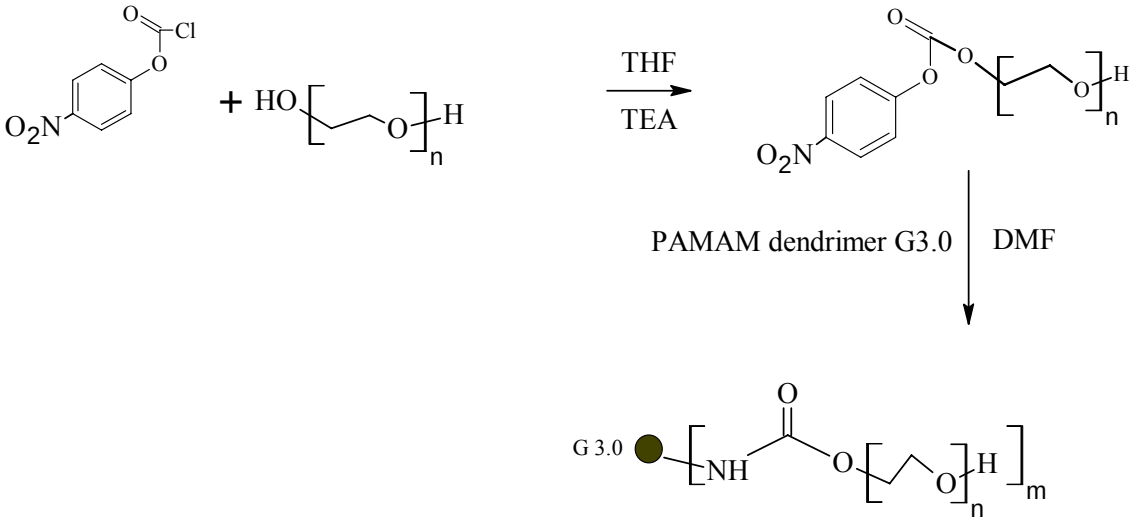
Name	Purpose
Weighing Balance	For measuring the required amount of materials
Eppendorf Centrifuge Model: 5415 D	For Centrifugation, separation of the suspended phase form liquid phase
Ultra Violet – Visible (UV-Vis) Spectrophotometer	Quantitative analytical tool for ninhydrin assay and release experiments.
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
Rotary Evaporator	For evaporating the solvents used in the experiment for obtaining the polymer
Scanning Electron microscope [ZESIS EVO50 SEM]	To take the SEM pictures for studying the internal structure and organization of Polymers
Fluorescent Microscope	Cell internalization assay
Hemacytometer	For cell counting in cell cytotoxicity assay
Binocular microscope	Cell studies

3.3 Synthesis

3.3.1 Conjugation of PEG to full generation PAMAM dendrimer G3.0

As illustrated in Figure 1, 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 ^{o}C 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. This solution was precipitated in 50 ml of ethyl ether and kept at -40 ^{o}C for further precipitation. The precipitate was collected and freeze dried with FTS [58]. 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 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 1. Conjugation of PEG to the dendrimer



The feeding molar ratio of OH-PEG-NPC/ dendrimer was reduced to 4:1 to prepare a lower degree of PEGylation on the dendrimer surface following the same procedure as described above.

3.3.2 Conjugation of PEG to half generation PAMAM dendrimer G3.5

Conjugation of PEG to half generation PAMAM G3.5 involved the activation of carboxyl (-COOH) groups of the dendrimer using DCC and DMAP. Prior to the reaction, 1µmol of PAMAM G3.5 was dried via rotary evaporation. The obtained dry

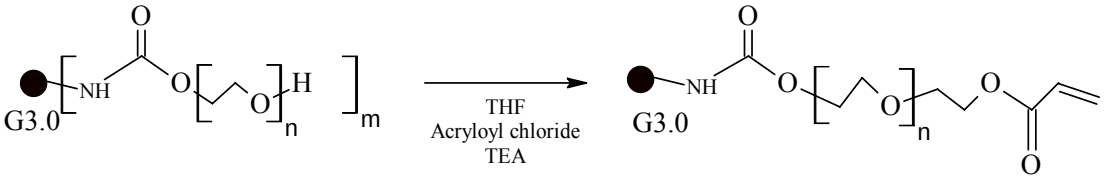
product was then dissolved in 1 ml of DI water. The solution was then acidified with 3 drops of 1 Normal HCl. The acidified solution was dried again by rotary evaporation and then re-dissolved in 2 ml of DMF. To this solution PEG diol was added, followed by the addition of DCC and DMAP where the feeding molar ratio of PEG diol: DCC: DMAP: G3.5 was 64:64:64:1. The solution was stirred for 24 hrs at 4 ^{o}C. After 24 hours the solution was added dropwise to cold ether and kept at -40 ^{o}C for 24 hrs. The precipitate was obtained by centrifugation. Dialysis was carried out to remove un-reacted DCC, PEG, and DMAP for further purification of the product. The product thus recovered was G3.5-PEG-OH. The degree of PEGylation was then determined by ^{1}H-NMR.

3.3.3 Conversion of free hydroxyl group of PEG to an acrylate group

As shown in Figure 2, 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 [59]. G3.5-PEG-OH was converted to G3.5-PEG-acrylate following the same procedure as described above.

Figure 2. Chemistry for introduction of a UV sensitive double bond to PEGylated G3.0



3.3.4 Labeling PAMAM dendrimer G3.0 with FITC

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 FITC labeled G3.0-PEG-acrylate.

3.3.5 Hydrogel formation

Three types of photoinitiators (i.e. DMPA, Irgacure, and Eosin Y) were evaluated in order to derive the best properties of hydrogels. The one that yielded sol-gel phase transition was then applied for hydrogel formation and was subjected to further characterization. Three different concentrations of PEG-acrylate (7.5, 20, and 40 wt %) were used for hydrogel formation. The concentration of acrylate was calculated using the following equation:

$$\left\langle \frac{m \times (MW\ of\ acrylate) \times (degree\ of\ PEGylation)}{(\ MW\ of\ PEGylated\ dendrimer\ )} \right\rangle / 100 = x\%\ (\textbf{Equation 1}),$$

where m= amount of polymer, x = pre determined weight % of polymer used for hydrogel formation (i.e. 7.5, 20, 40 wt %)

For each sample preparation various amount of polymer, with constant acrylate concentration was dissolved in 100 µL of DI water. To this 5 µL solution of photoinitiator system containing (Eosin Y 0.1%, Triethanolamine [TEOA] 40 % and 1 vinyl 2 Pyrrolidinone [NVP] 4% by wt) [60] was added. Hydrogel formation was realized by subjecting a mixture solution of polymer and photoinitiator to UV radiation [325 nm] for 30 min or by curing it under lamp light or a combination of both UV and lamp light.

Table 5. Initial screening of photoinitiators and conditions for hydrogel formation

Polymer*	Photoinitiator	Water (μL)	Duration of UV light exposure (mins)
G3-[PEG1500-acrylate]$_{29}$	DMPA 2mg	100	10
G3-[PEG 6000-acrylate]$_{23}$	DMPA 2mg	100	10
G3-[PEG 12000-acrylate]$_{28}$	DMPA 2mg	100	10
G3-[PEG 1500-acrylate]$_{29}$	DMPA 2mg	100	30
G3-[PEG 6000-acrylate]$_{23}$	DMPA 2mg	100	30
G3-[PEG 12000-acrylate]$_{28}$	DMPA 2mg	100	30
G3-[PEG 1500-acrylate]$_{29}$	Irgacure 2mg	100	10
G3-[PEG 6000-acrylate]$_{23}$	Irgacure 2mg	100	10
G3-[PEG 12000-acrylate]$_{28}$	Irgacure 2mg	100	10
Linear Peg 1500-acrylate	DMPA 2 mg , Irgacure, EosinY system	100	10
G3-[PEG1500-acrylate]$_{29}$	Eosin Y system 5 μL	100	30
G3-[PEG 6000-acrylate]$_{23}$	Eosin Y system 5 μL	100	30
G3-[PEG 12000-acrylate]$_{28}$	Eosin Y system 5 μL	100	30

*= 7.5%wt (Concentration of polymer used for hydrogel formation)

G3.0-[PEG # -acrylate]$_{*}$: #, molecular weight of PEG; $_{*}$, degree of PEGylation; Eosin Y system contained Eosin Y 0.1% by wt, Triethanolamine [TEOA] 40 % by wt and 1 vinyl 2 Pyrrolidinone [NVP] 4% by wt.

3.3.6 Formation of hydrogels composed of linear PEG only

For comparative study one of the two hydroxyl end groups of PEG diol [MW 1500] was modified to form OH-PEG 1500-acrylate using the same procedure as described for modification of PAMAM-PEG-OH in section 3.3.3. The derived product was used to form hydrogel utilizing the aforementioned procedure in section 3.3.5. Different concentrations such as 2 mg/ 100 μL, 8.2 mg/ 100 μL , 10mg/100 μL of OH-PEG 1500-

acrylate were utilized for forming hydrogels. In addition the minimum concentration at which gelation occurred was determined.

3.3.7 Semi-interpenetrating networks

As the formation of hydrogels from low PEGylated dendrimers was not observed, semi IPNs were formulated as an alternative strategy. Gelatin, liner OH-PEG 1500-acrylate, and linear PEG diol were chosen as a second polymer to form semi IPN with low PEGylated dendrimers.

3.3.7.1 Semi-IPN composed of low PEGylated dendrimer and gelatin

The molar concentration of acrylate in G3.0-PEG-acrylate with a low degree of PEGylation used for forming semi IPNs was kept the same as used in section 3.3.5. 100mg of gelatin was dissolved in 1 ml of water and heated at 50 °C for 5 minutes to dissolve the gelatin completely. This solution was then mixed with a solution containing low PEGylated dendrimer in 100 µL of water. To this 5 µL of photo initiator solution was added. This solution was subjected to UV radiation for 30 minutes.

3.3.7.2 Semi-IPN composed of low PEGylated dendrimer and linear PEG 1500-acrylate

The molar concentration of acrylate in PAMAM-PEG-acrylate with a low degree of PEGylation was kept the same as used in section 3.3.5. To this solution 2 mg of linear PEG-acrylate was added. The concentration of the linear PEG-acrylate used for forming semi IPN was much lower than the minimum concentration of linear PEG-acrylate alone for forming hydrogels. Both polymers were dissolved in 100 μL of DI water. To this solution 5 μL of photoinitiator solution was added. This solution was subjected to UV radiation for 30 minutes.

3.3.7.3 Semi-IPN composed of low PEGylated dendrimer and linear PEG diol

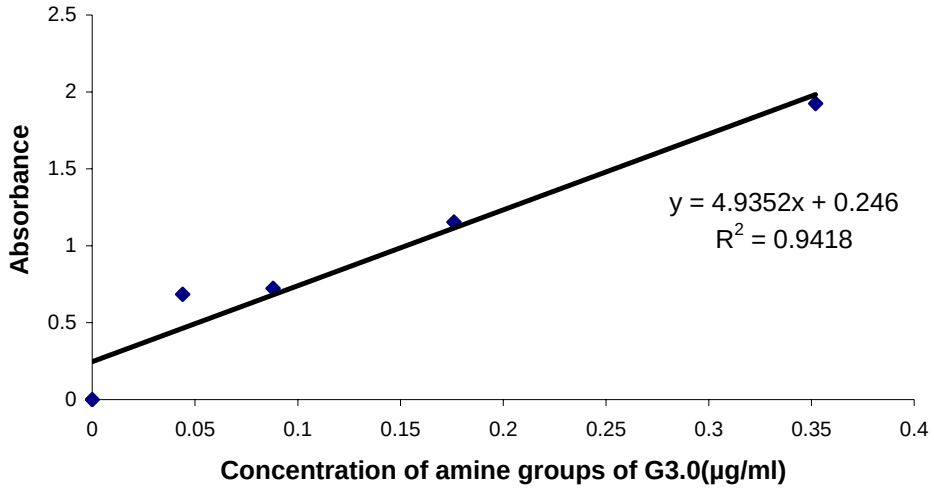
Dendrimer-PEG -acrylate with low degree of PEGylation was mixed with linear PEG diol (MW 1500) in 100 μL of DI water. To this solution 5 μL of photo initiator solution was added. The solution was then subjected to UV radiation for 30 minutes.

3.4 Characterization

3.4.1 Ninhydrin Assay

The remaining amine groups on the surface of dendrimer were quantified by ninhydrin assay for indirect determination of the degree of PEGylation of dendrimer. The stock solution of ninhydrin was prepared by dissolving 70 mg of ninhydrin in 20 ml of ethanol. A sample (1 mg) was dissolved in 1 ml of ethanol or water and then mixed with 1 ml of ninhydrin stock solution. The mixture was heated at 85 ^{o}C for 5 mins and then cooled down to room temperature. The absorbance, at 570 nm was measured with UV-Vis Spectroscopy. Then the concentration of the amine group was calculated, based on standard curve of dendrimer (Figure 3).

Figure 3. Standard curve of PAMAM dendrimer G3.0



The degree of PEGylation was calculated using the following equation:

$$\left\langle \left[\frac{1\,mg\ PEGylated\ G3.0}{(MW\ of\ G3.0)+n(MW\ of\ PEG)} \right] / \begin{pmatrix} Volume\,of \\ sample \end{pmatrix} \right\rangle \times 100 \times (32-n) = \begin{pmatrix} Concentration\,of \\ \text{amine}\ groups \end{pmatrix}$$

(Equation 2)

where n is degree of PEGylation and G3.0 dendrimer has 32 amine groups.

3.4.2 ^{1}H-NMR

The ^{1}H-NMR spectra of dendrimer derivatives were recorded on a 400 MHz NMR spectrometer (Inova-400). The solvent was deuterium water (D_2O), which has a chemical shift of 4.8 ppm. The ^{1}H-NMR results were processed using Spinworks software. As an alternative method, the degree of PEGylation of dendrimer was determined based on the integrals of corresponding proton peaks.

$$\frac{4n \times X}{m} = \frac{A1}{A2}$$ (**Equation 3**)

- 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 Water swelling studies

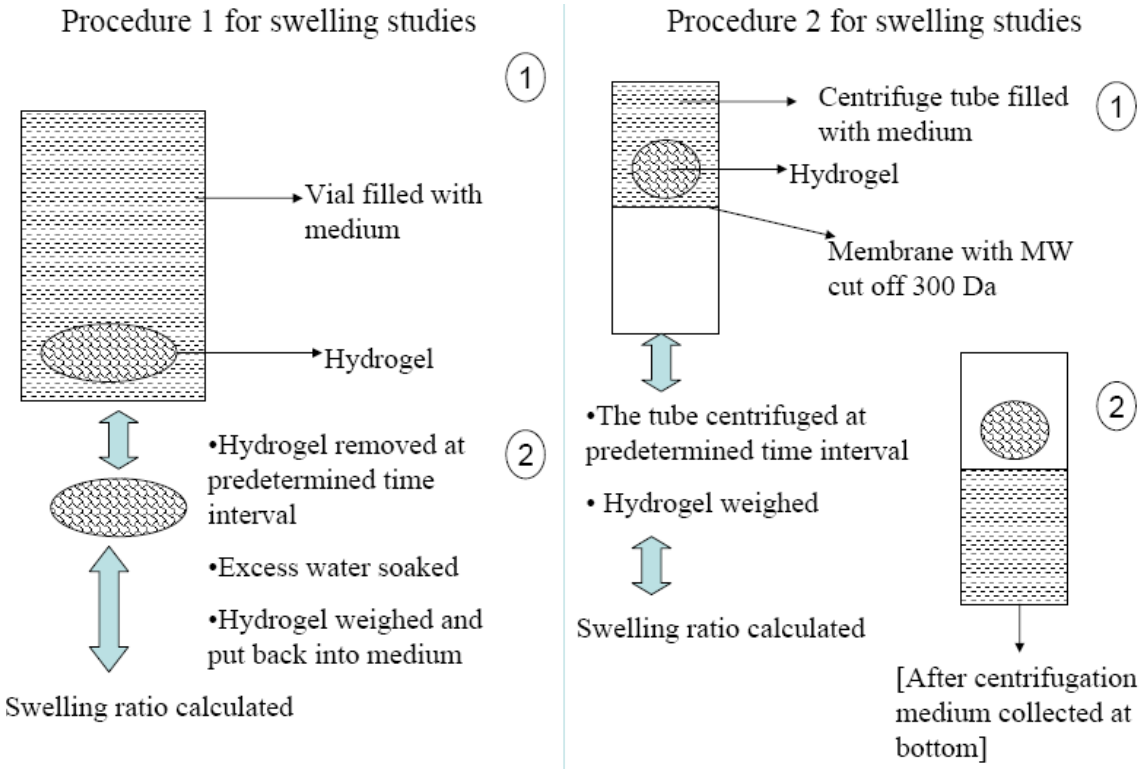
The prepared hydrogel were subjected to swelling test to evaluate the equilibrium water content (EWC) within the network. Water swelling experiments were conducted at room temperature at different pHs (i.e., 4.4, 2, 7.4, and 10). Prior to evaluation of equilibrium water content or calculation of the swelling ratio, the hydrogels were dried. Hydrogel samples were accurately weighed prior to immersion into the swelling media. The hydrogel samples were taken out periodically from the swelling media, blotted dry with an absorbent tissue and weighed. Each water swelling test was carried out over a period of 24 hours.

The second method utilized centrifuge tubes with a membrane having a molecular weight cut off of 300 Da. As illustrated in Figure 4, each hydrogel sample was placed in the upper chamber of the tube having membrane and incubated in the medium. These centrifuge tubes containing hydrogel and the medium were centrifuged at predetermined time points. The medium was collected at the bottom tube. The hydrogel was weighed and swelling ratio calculated. The hydrogels were put back in the tubes after weighing and same procedure was carried out for 24 hours of incubation. The swelling ratio of hydrogel was calculated with the following formula;

$$Swelling\ ratio\,\% = \frac{(Ws - Wd)}{Wd} \times 100$$ **(Equation 4),**

where Ws = weight of swollen hydrogel, Wd = Weight of dry hydrogel.

Figure 4. Schematic representation of experimental setup for water swelling studies displaying two methods used



3.4.4 Study of degradation of hydrogels

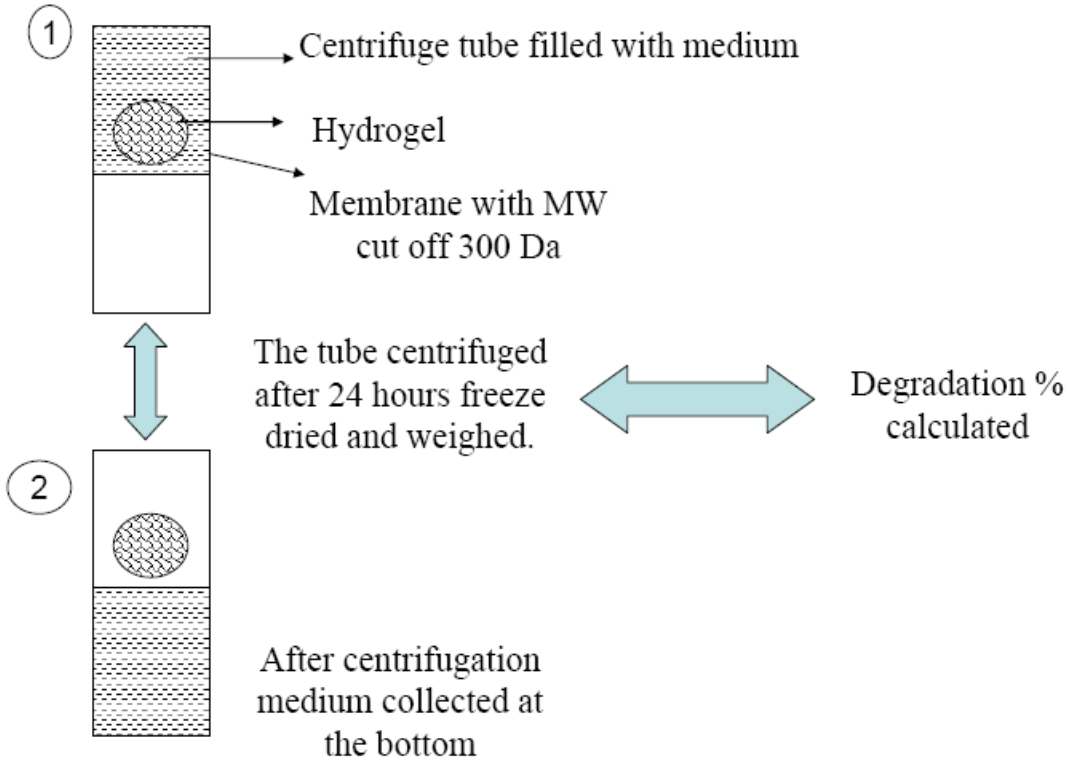
It was observed that hydrogel degraded to a certain extent during water swelling studies. To characterize the degradation of hydrogel, samples were weighed and incubated in medium (i.e. neutral, pH of 2, 4.4, 7.4, and 10) in centrifuge tubes. As illustrated in Figure 5, centrifugation was carried out after 24 hrs of incubation. The supernatant was poured out and subsequently the sample from the bottom portion of centrifuge tube was

weighed and utilized to determine swelling ratio. The sample was then freeze dried to make sure it was completely free of absorbed medium and weighed again to determine the degradation. The degradation extent was calculated using the following equation;

$$Degradation\,\% = \frac{(Dw - FDw)}{Dw} \times 100 \quad \textbf{(Equation 5)},$$

Where *Dw*= Weight of dry hydrogel prior to immersing in medium, *FDw* = Weight of freeze dried hydrogel after 24 hrs of incubation in medium.

Figure 5 Schematic representation of experimental set up for degradation studies



3.4.5 Sol-gel phase transition studies

In order to minimize the exposure of UV radiation for hydrogel formation, a combination of regular day light and UV radiation was studied. This study was carried out to determine the conversion from sol to gel phase over a period of time for dendrimer based hydrogel. G3.0-$[PEG\ 12000]_{28}$ was used in this study. For this 7.5 wt % polymer was dissolved in 100 µL of distilled water. To this solution, 5 µL of photoinitiator Eosin Y system was added. 12 sample solutions were prepared. Then these solutions were allowed to cure under day light for 24 hrs, 48 hrs, 72 hrs and 1 week; three samples for each time period of curing. After 24 hrs, three samples were subjected to UV light for one minute, 5 minutes, and 10 minutes, respectively. The vials were inverted to determine the flow or no flow condition and the time after which the flow was seen. Similarly the samples exposed to regular day light for 48, 72 hrs, and 1 week were subjected to 1, 5, and 10 minutes of UV exposure and tube inversion was done to determine the sol-gel transition phase.

3.4.6 Scanning electron microscopy (SEM)

To take SEM pictures, sample hydrogel was first mounted on the stub over double sided sticky carbon paper, and then the stub with mounted sample was coated with gold for 2 min and 40 seconds at 30 miliamps. This gold plated stub was then placed inside the

SEM chamber under high vacuum. The chamber is equipped with probes for visualization of sample placed in the chamber.

3.4.7 Analysis of drug release kinetics

To understand the mechanism of release of the active agent from the prepared hydrogels, drug Cyclosporine A, which is sparingly soluble in water, was used. Drug loading was done in two ways. One way used ethanol as a medium to form hydrogel instead of DI water as Cyclosporine A is readily soluble in ethanol. In this method drug was dissolved in ethanol with polymer (G3.0-[PEG 12000-acrylate] $_3$ linear PEG 1500-acrylate) and the solution was stirred vigorously. This mixture of polymer and drug was mixed with photoinitiator solution and exposed to UV radiation. The second way based on water for forming drug incorporated hydrogel is described as follows. First the polymer was dissolved in 100 μL DI water. To this solution excess mount of cyclosporine A was added. This solution was vortexed vigorously and incubated for 24 hours. After 24 hours the solution was centrifuged to remove the solids and the supernatant (saturated with cyclosporine A) collected and mixed with photoinitiator solution, then exposed to UV radiation. It was assumed that the drug would be incorporated within the core of dendrimer. Similar procedure was followed for half generation dendrimer (G3.5-[PEG 1500-acrylate] $_{43}$). Linear PEG 1500-acrylate hydrogel was used as control. These hydrogel were placed in dialysis bag, and then immersed in 100 ml medium at different

pHs (i.e., 2, 7.4, and 10) for 24 hours covered with parafilm and stirring constantly. Samples were taken from this solution at predetermined time intervals and analyzed using UV–Vis spectrophotometer (Figure 6). The absorbance measured with UV-Vis spectrophotometer was compared with the standard curve of cyclosporine A and the concentration of the drug was determined (Figure 7 and 8).

Figure 6 Schematic representation of experimental set up for performing drug release kinetics

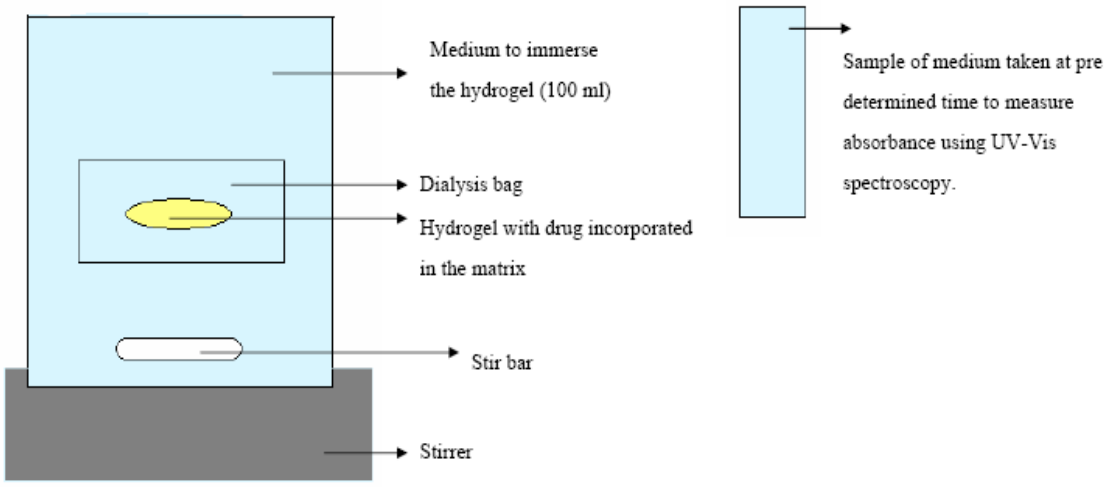


Figure 7. Standard curve of cyclosporine A (water as solvent)

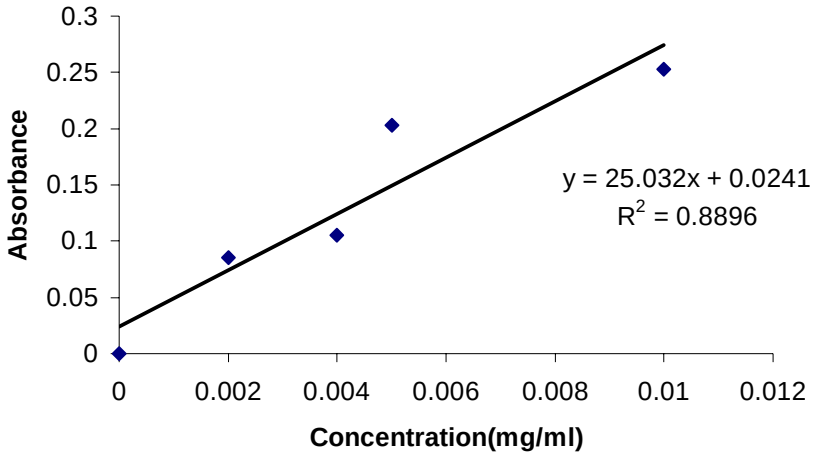
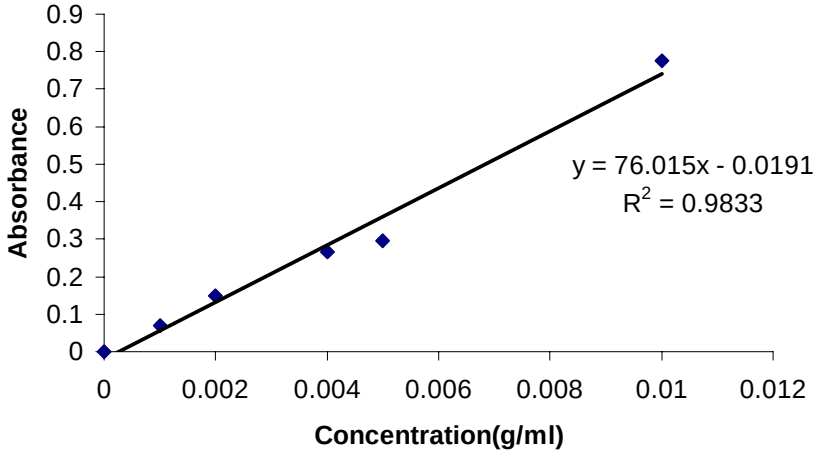


Figure 8. Standard curve of cyclosporine A (ethanol as solvent)



The total amount of drug released from each hydrogel sample was compared with the calculated amount of incorporated drug by measuring the absorbance of the solution of polymer and cyclosporine A prior to hydrogel formation.

Drug release kinetics was plotted by calculating percentile drug release against a period of time. Drug release percentile was calculated as follows:

$$DR\% = \frac{C_t}{C_i} \times 100 \quad \textbf{(Equation 6)}$$

Where DR% = drug release %, C_t= Concentration of drug released at time (t), and C_i = Concentration of drug at initial time within the hydrogel.

3.4.8 Cytotoxicity evaluation

The cytotoxicity of the FITC-conjugated G3.0-PEG was evaluated *in vitro* using cell line RAW264 mouse macrophages. RAW264 mouse macrophages (1×10^3 cells/well) were seeded in a 24-well cell culture plate at 37°C in 1 ml of medium (DMEM medium supplemented with 10% fetal calf serum, 100 UI/ml penicillin-streptomycin) in an atmosphere of 10% CO_2. After 24 h, the culture medium was replaced and different amounts of FITC-G3.0-[PEG12000-acrylate]$_{28}$ (*Mw*= 34685) and cross-linked FITC-G3.0-[PEG12000- acrylate]$_{28}$ (*Mw*= 34685) were added. Their final concentrations were 0.2, 2, 20, 50, or 100 µM. The culture plate was then incubated at 37°C in a tissue culture incubator for 2 days. After incubation at 37°C for 2 days the medium was aspirated and 200 µL of trypsin solution was added to each well to prepare cell suspension solution. Then the cell suspension solution together with former medium was centrifuged at 3000 rpm for 3min and the supernatant was discarded. The cells

were re-suspended in 0.1 ml of PBS or serum-free complete medium and to it 0.1 ml of 0.4% trypan blue solution added. The mixture was allowed to incubate 3 min at room temperature. Then a drop of trypan blue/cell mixture was placed onto a hemacytometer. The hemacytometer was then used to count cells. The unstained (viable) cells were then counted.

$$Cell\,Viability\,\% = \frac{Total\ number\ of\ viable\ cells\ per\ ml\ of\ liquid}{Total\ \ number\ of\ cells\ per\ ml\ of\ liquid} \times 100 \quad (\textbf{Equation 7})$$

3.4.9 Cell internalization studies

RAW264 mouse macrophages (1×10^3 cells/well) were seeded in a 24-well cell culture plate at 37°C in 1 ml medium (DMEM medium supplemented with 10% fetal calf serum, 100 UI/ml penicillin-streptomycin) in an atmosphere of 10% CO_2.

After 24 h, the culture medium was replaced and different concentrations (0.2, 2, 20, 50 or 100 μM) of FITC-G3.0-[PEG 12000-acrylate]$_{28}$ (*MW*= 34685) and cross-linked FITC-G3.0-[PEG 12000- acrylate]$_{28}$ (*MW*= 34685) were added to the relative medium as different groups. After that, in the time point of 15 min, 30 min, 60min, and 24h, medium was replaced by new medium for a group of cells. Fluorescence photos were taken to determine cell internalization.

CHAPTER 4 Results and Discussion

4 Results and Discussion

4.1 Calculation of degree of PEGylation of dendrimer

To confirm and calculate the degree of PEGylation of dendrimer (i.e. the average number of PEG chains per dendrimer) as well as the molecular weight of PEGylated dendrimers ninhydrin assay and ^{1}H-NMR were applied.

4.1.1 Ninhydrin assay results

The degree of PEGylation was indirectly determined by assessing the number of un-conjugated amine groups on the dendrimer surface based on ninhydrin. The molecular weight was calculated using following equation.

$$\left(MW\ of\ PEGylated\ \ dendrimer \right) = MW\ of\ dendrimer + \left[\left(MW\ of\ PEG \right) \times \left(\text{degree}\ of\ PEGylation \right) \right]$$

(**Equation 8)**

A high degree of PEGylation of PAMAM G3.0 dendrimer was achieved regardless of PEG length when the feeding molar ratio of PEG to dendrimer was 32:1. The degree of

PEGylation was 29 for PEG 1500 (i.e. G3.0-[PEG 1500]$_{29}$), 23 for PEG 6000 (i.e. G3.0-[PEG 6000]$_{23}$), and 28 for PEG 12000 (i.e. G3.0-[PEG 12000]$_{28}$). The corresponding molecular weights of the conjugates were 50460, 144960, and 354960 respectively. However, the degree of PEGylation was estimated to be higher than feeding ratio used for conjugation when the feeding molar ratio of PEG to dendrimer was low, i.e., 4:1. This may be caused by steric hindrance of PEG, which shields the amine groups on the surface of dendrimer. Therefore when ninhydrin assay is performed, the number of amine groups available for reaction with ninhydrin tends to be lower than what it should be, thus leading to estimation of higher loading degree. To overcome this limitation, ^{1}H-NMR was applied as an alternative method.

4.1.2 ^{1}H-NMR

The ^{1}H-NMR spectroscopy revealed a very similar degree of PEGylation of dendrimer when the feeding molar ratio of PEG to dendrimer was high, i.e., 32:1 (Figure 9, 11, and 13). As the steric hindrance of PEG affects the ninhydrin assay results, ^{1}H-NMR results are more accurate than the ninhydrin assay-based results. When the feeding molar ratio of PEG to PAMAM G3.0 was 4:1, the actual degree of PEGylation was 4 for PEG 1500 (i.e., G3.0-[PEG 1500]$_{4}$, Figure 10), 3 for PEG 6000 (i.e., G3.0-[PEG 6000]$_{3}$, Figure 12), and 3 for PEG 12000 (i.e., G3.0-[PEG 12000]$_{3}$, Figure 14). The degree of PEGylation of half generation PAMAM dendrimers can only be estimated by ^{1}H-NMR

as half generation dendrimers do not have amine groups. The degree of PEGylation of G3.5 PAMAM dendrimer was 43, (i.e., G3.5-[PEG 1500]$_{43}$, Figure 15) when the molar feeding ratio of PEG to dendrimer was 64:1.

Figure 9. ^{1}H-NMR spectrum of G3.0-[PEG 1500]$_{29}$

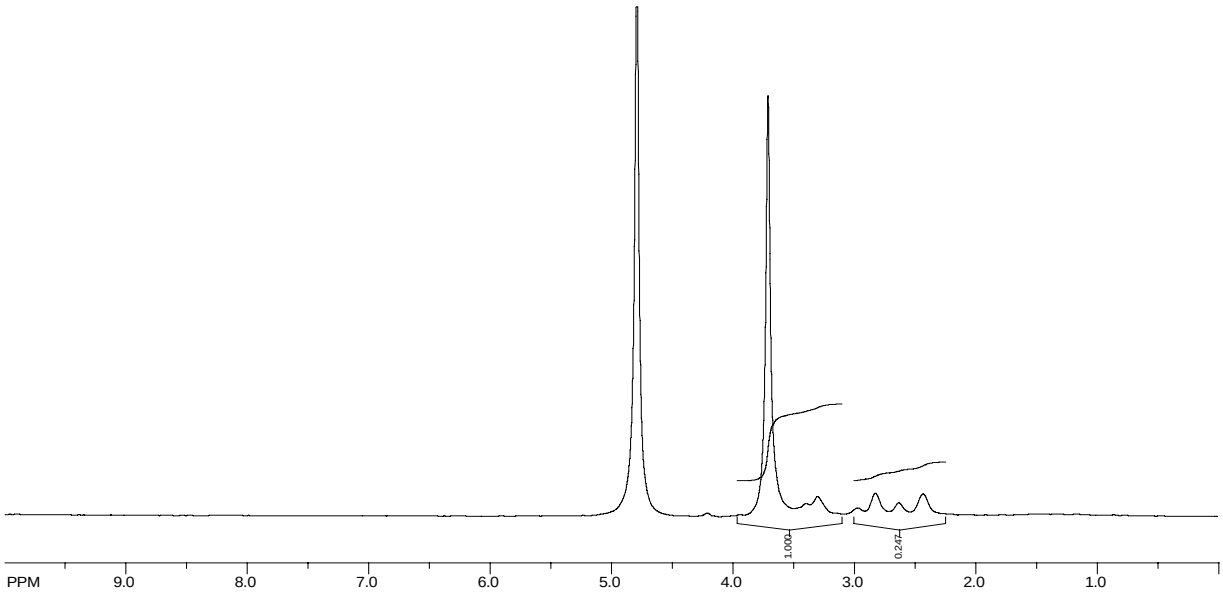


Figure 10. ^{1}H-NMR spectrum of G3.0-[PEG 1500]$_4$

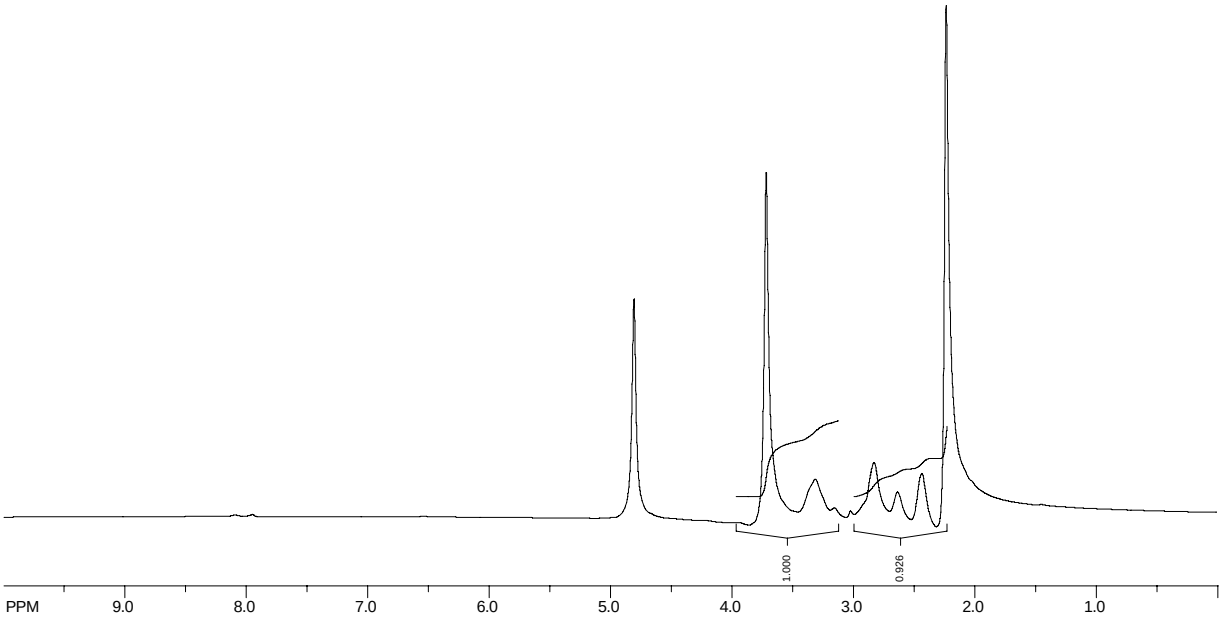


Figure 11. ^{1}H-NMR spectrum of G3.0-[PEG 6000]$_{23}$

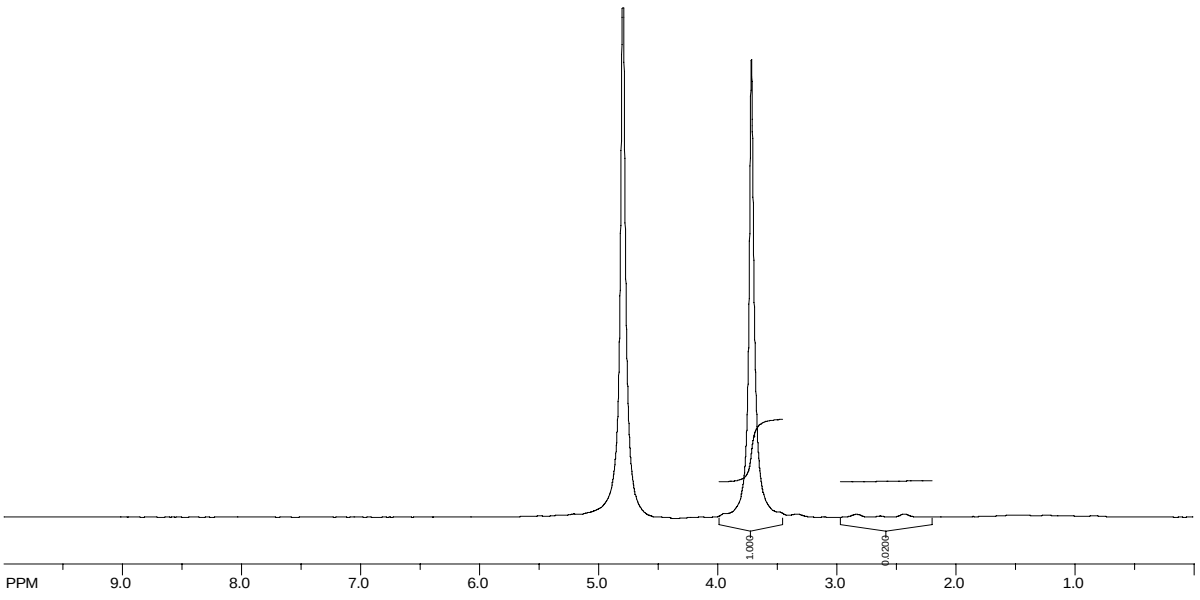


Figure 12. ^{1}H-NMR spectrum of G3.0-[PEG 6000]$_3$

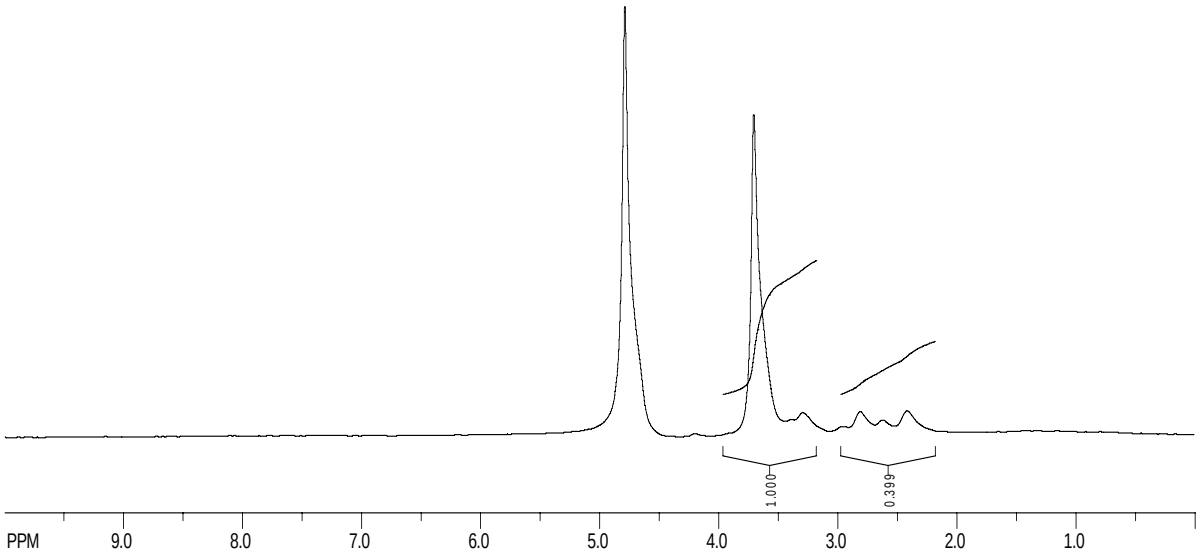


Figure 13. ^{1}H-NMR spectrum of G3.0-[PEG 12000]$_{28}$

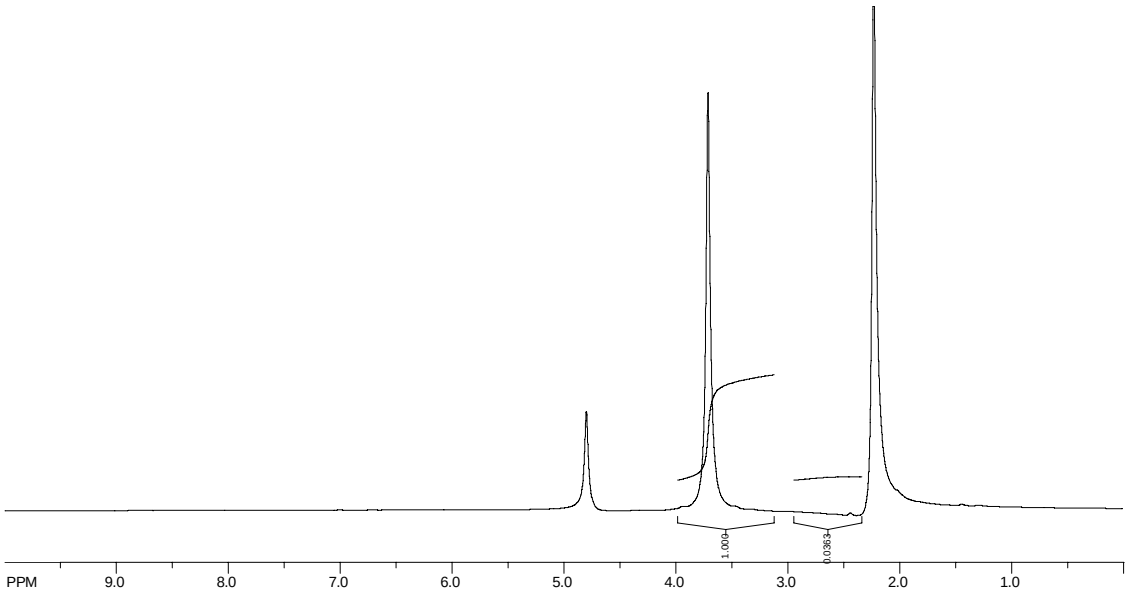


Figure 14. ^{1}H-NMR spectrum of G3.0-[PEG 12000]$_3$

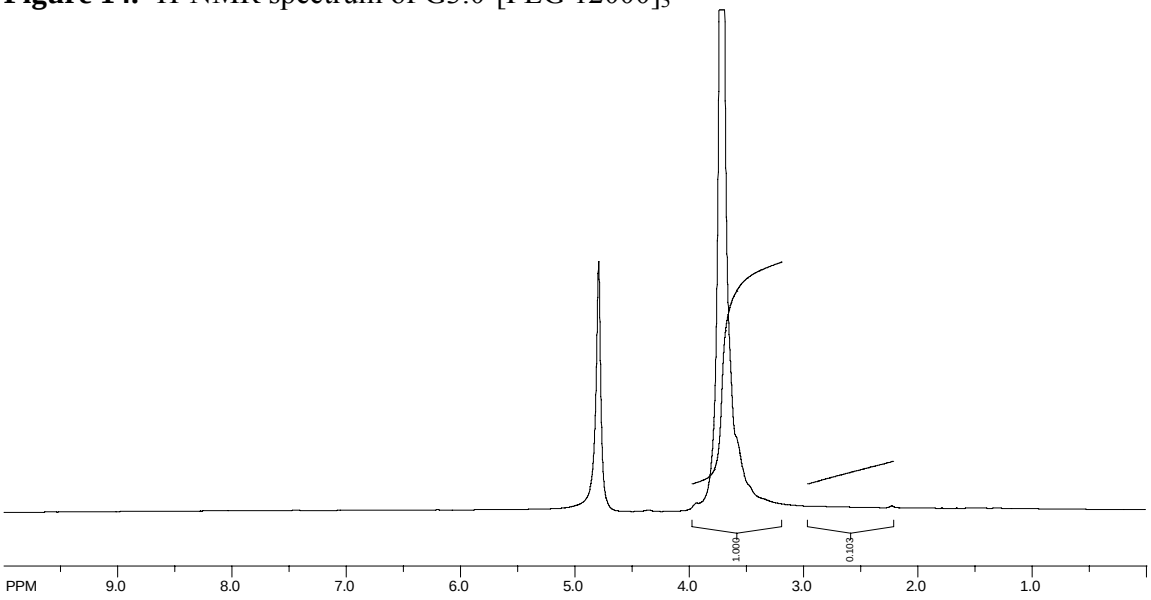
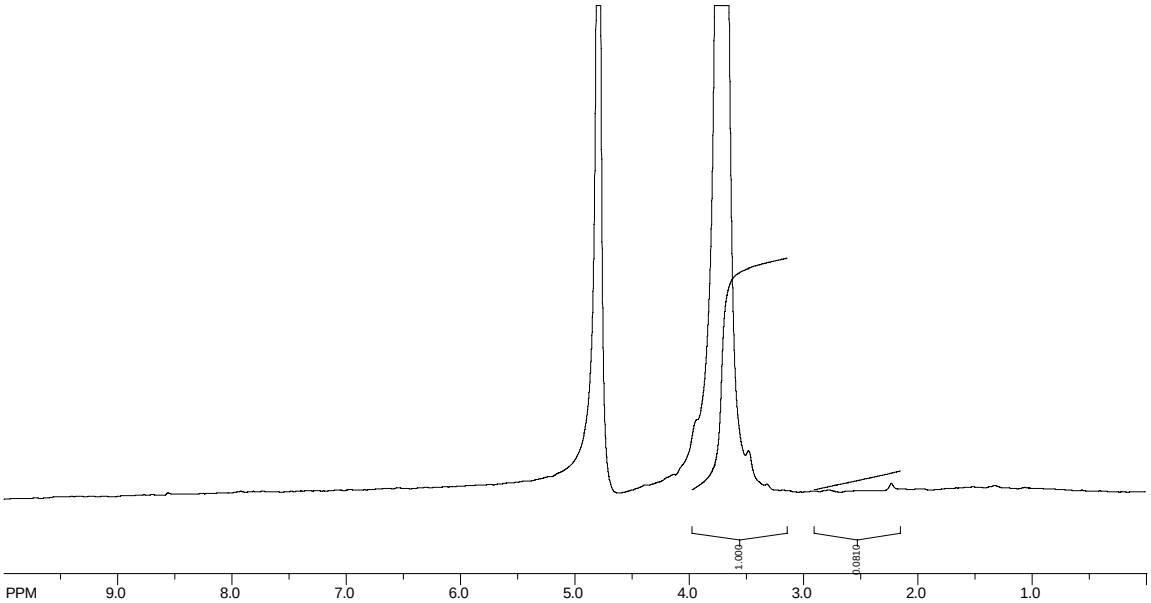


Figure 15. ^{1}H-NMR spectrum of G3.5-[PEG 1500]$_{43}$



4.2 Observation on hydrogel formation

In this study three different photoinitiator, i.e., DMPA, Irgacure, and Eosin Y system, were investigated for hydrogel formation. Linear PEG 1500-acrylate was used as control. PEGylated dendrimers were dissolved in water to have the same molar concentration of acrylate groups as linear PEG-acrylate had. All of the three photoinitiators triggered hydrogel formation based on linear PEG 1500-acrylate control under the conditions specified in the experiments. We observed that PEGylated dendrimers did not form hydrogels with the use of DMPA or Irgacure. In contrast PEGylated dendrimers formed hydrogels using the Eosin Y photoinitiator system. Hydrogel formation was also affected by PEG length in case of high PEGylated full generation dendrimer; higher the PEG length, higher the viscosity of the solution. Further the gelation process was shortened when compared to lower PEG lengths under the same conditions. As low PEGylated dendrimers alone were unable to form hydrogel, a secondary polymer (i.e., gelatin, PEG 1500-acrylate, and PEG 1500 diol) was applied to facilitate hydrogel formation of low PEGylated dendrimers. The observation on the morphology of hydrogel is enumerated in Table 6.

Table 6. Observation on hydrogel formation (7.5 % by wt concentration of polymer, 100 μL of DI water, Eosin Y 0.1% by wt, TEOA 40 % by wt, NVP 4 % by wt, 30 min UV radiation)

	Hydrogel	Polymerization condition after UV radiation for 30 mins [325 nm]
A	G3-[PEG1500-acrylate]$_{29}$	Thick viscous liquid, clear, pale yellowish in color
B	G3-[PEG 6000-acrylate]$_{23}$	Thick viscous liquid, clear, pale yellowish
C	G3-[PEG 12000-acrylate]$_{28}$	Soft, sticky, clear and pale yellowish hydrogel
D	G3-[PEG 1500-acrylate]$_4$	No gel formation observed, the viscosity did not change, clear solution
E	G3-[PEG 6000-acrylate]$_3$	No gel formation observed, the viscosity did not change, clear solution
F	G3-[PEG 12000-acrylate]$_3$	No gel formation observed, the viscosity did not change, clear solution
G	G3-[PEG 12000-acrylate]$_3$ + linear PEG 1500-acrylate	Soft, sticky, clear and pale yellowish hydrogel
H	Linear PEG 1500-acrylate	Soft , clear , pale yellowish hydrogel
I	G3-[PEG 12000-acrylate]$_3$ + gelatin	Soft, sticky, clear and pale yellowish hydrogel
J	G3-[PEG 12000-acrylate]$_3$ + linear PEG 1500	No gel formation observed, the viscosity did not change, clear solution
K	G3.5-[PEG 1500-acrylate]$_{43}$	Soft, flexible, clear hydrogel

Figure 16. Appearance of G3-[PEG 12000-acrylate]$_{28}$ hydrogel after exposure to UV radiation

Incorporation of linear PEG 1500-acrylate led to formation of semi IPN based on low PEGylated dendrimers. It is believed that incorporation of linear PEG 1500-acrylate provided more acrylate groups for crosslinking reaction, thus strengthening the network formation. To understand the mechanism of hydrogel formation, the concentration of linear PEG-acrylate was increased within the semi IPN. It was seen that with higher concentrations of linear PEG-acrylate hydrogels improved the structural stability of the network. Incorporation of gelatin also helped in IPN formation based on low PEGylated dendrimers. Although the mechanism of gelatin facilitating hydrogel formation remains to be elucidated, it was believed that addition of gelatin helped to link the dendrimer molecules together by reducing the void spaces. However, PEG diol along with low

PEGylated dendrimers did not undergo hydrogel formation. Neither gelatin nor PEG diol alone is capable of forming hydrogel at the given concentration.

4.2.1 Sol-gel phase transition studies

Hydrogel formation using these three different photoinitiators was evaluated in terms of their network structure and sol-gel phase transition. Hydrogel formation was best realized using Eosin Y photoinitiator. It was observed that 30 minutes of UV exposure was needed for hydrogel formation. The exposure of UV radiation may need to be reduced for *in situ* hydrogel formation. In an attempt to cut down UV exposure, solutions of the polymer and photoinitiator were cured under regular day light for different time periods first, followed by UV exposure.

Table 7. Sol-gel phase transition of G3.0-[PEG 12000-acrylate]$_{28}$

Time cured in day-light	24 hours			48 hours			72 hrs			1 week		
Time of UV exposure	1 Min	5 min	10 min	1 min	5 min	10 min	1 min	5 min	10 min	1 min	5 min	10 Min
Flow results after tube inversion	--	--	-	(+)	(+)	+	+	+	++	++	++	++

Instant Flow = -- , Flow after 5 seconds = - , Flow after 10 seconds = (+) , Flow after 20 second = + , Flow after 30 seconds = ++

Table 7 shows the results of Sol-gel phase transition studies. It was observed that UV exposure can be reduced to about 10 minutes with the utilization of combination of two sources of curing, regular day light and UV radiation. When the mixture of polymer and photoinitiator was allowed to cure in regular day light for a longer time period hydrogel formation can be realized with the UV exposure reduced to between 1 and 10 minutes. Linear PEG-acrylate was used as control, hydrogel formation was observed after 24 hours of curing in regular day light without any UV exposure.

4.3 Effect of PEG length on hydrogel morphology

Figure 17. SEM picture of G3.0-[PEG1500-acrylate]$_{29}$ hydrogel (magnification 1.97 K)

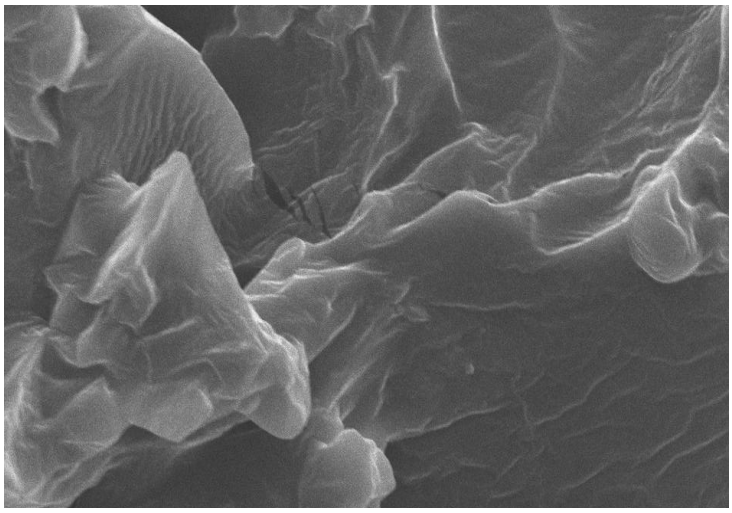


Figure 18. SEM picture of G3-[PEG 6000-acrylate] $_{23}$ hydrogel (magnification 1.97 K)

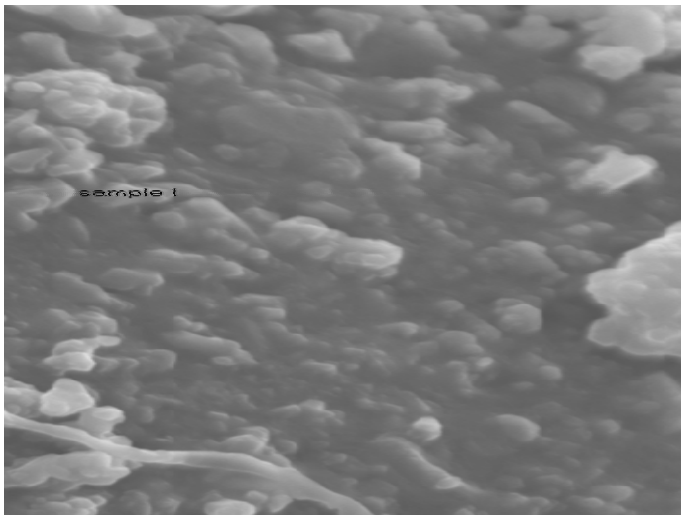


Figure 19. SEM picture of G3-[PEG 12000-acrylate] $_{28}$ hydrogel (magnification 1.97 K)

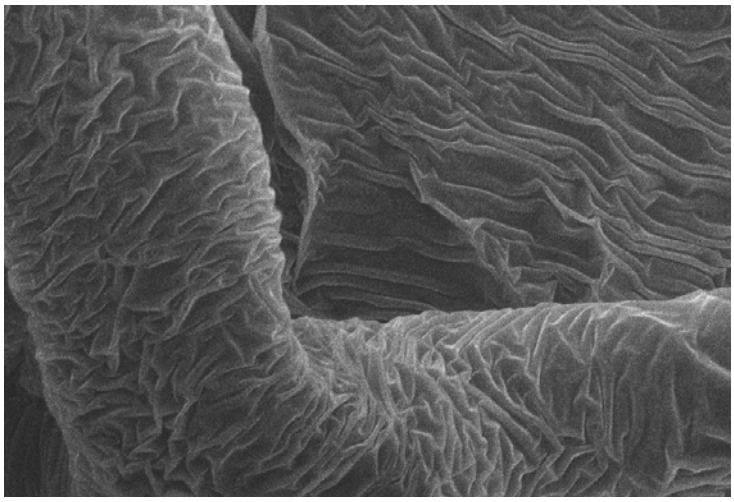
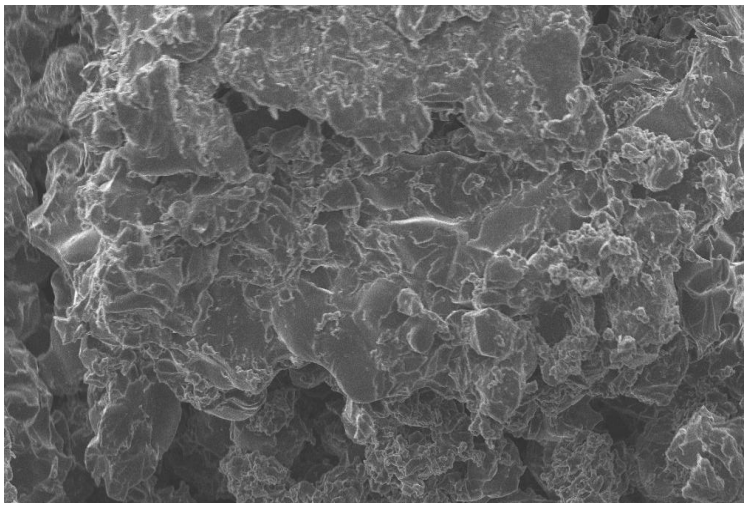


Figure 20. SEM picture of linear PEG 1500-acrylate hydrogel (magnification 1.97 K)



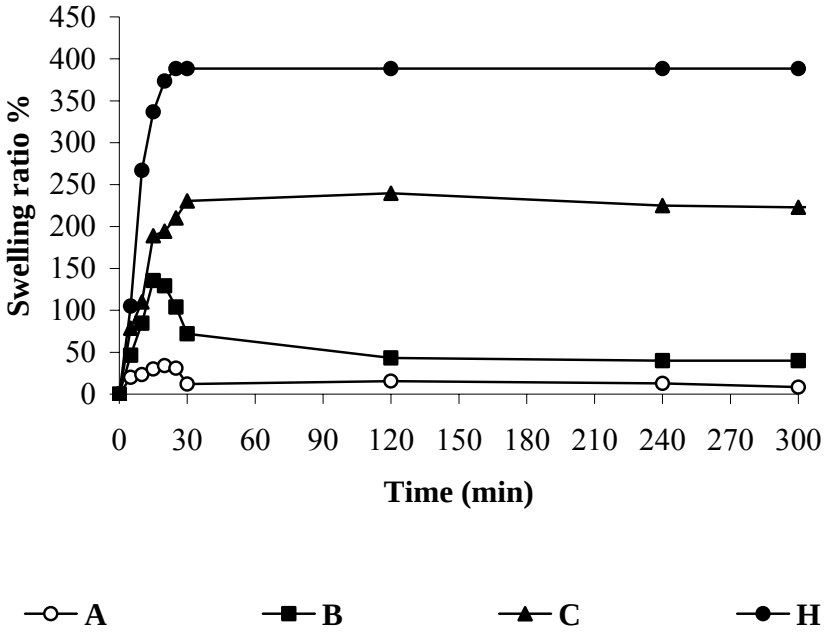
The SEM pictures of hydrogels were taken to determine the size of particles. The images were taken at a magnification of 1.97 K. At this low magnification we were unable to reach nanoscale to quantify the size of dendrimer within the hydrogel network. Due to the gluey stick characteristic of hydrogel it was not possible to visualize these hydrogels at higher magnification. However we still can see the microscopic difference in morphology of hydrogels due to PEG lengths variations.

4.4 Observation on water swelling studies

The water swelling results are presented in Figures 21-23. We found that polymer composition and pH affected the swelling behavior of the hydrogels. After 24 hours of incubation the swelling ratio remained constant for all the samples analyzed. As seen in Figure 21 G3.0-$[PEG\ 1500]_{29}$ reached the maximum swelling ratio (30 %) within 15

minutes of incubation and then its swelling ratio declined to about 3 % after 6 hours and remained constant thereafter. The maximum swelling ratio for G3.0-[Peg 6000]$_{23}$ was 135.5 % in about 15 minutes and declined to 40 % in around 6 hours and remained constant. G3.0-[PEG 12000]$_{28}$ reached its maximum swelling ratio of 239.64 % in 2 hours and remained quite constant when observed a period of 24 hours of incubation. The control linear PEG 1500-acrylate hydrogel showed a maximum swelling ratio of 338.33 %. Longer PEG favors a higher swelling ratio and helps the network maintain a constant swelling ratio as compared to lower PEG lengths. Hydrogels based on short PEG lengths disintegrated upon a short exposure to aqueous swelling medium. Also they reached equilibrium faster and most portion of the hydrogel degraded within a short period of time (i.e. 2-3 hours). Therefore, the low swelling ratio was probably due to mass loss.

Figure 21. Swelling ratio of PAMAM dendrimer G3.0 conjugated with different PEG lengths

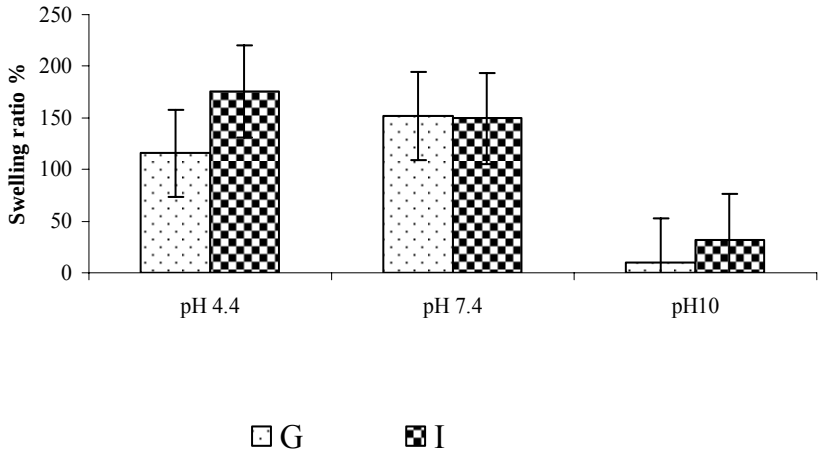


A= G3-[PEG 1500-acrylate]$_{29}$, **B**= G3-[PEG 6000-acrylate]$_{23}$, **C**= G3-[PEG 12000-acrylate]$_{28}$ + linear PEG 1500 acrylate, **H** = Linear PEG 1500-acrylate

It was observed that low PEGylated dendrimers were unable to form hydrogel. Therefore forming IPNs was attempted by combining low PEGylated dendrimer with linear PEG-acrylate, gelatin, or linear PEG diol. Water swelling studies showed that these IPNs displayed pH sensitivity to a certain extent. Full generation dendrimers have amine groups on their surface, in case of low PEGylated dendrimer (PEG 12000) these surface groups conduce sensitivity to the hydrogel. Figure 22-23 shows that the IPN hydrogel reached its swelling equilibrium within 2-3 hours and remained constant thereafter as seen from the Figure 22. It was observed that both IPN samples G and I had a lower swelling ratio at higher pH (i.e.pH10) than at lower pHs (pH 4.4 and 7.4).

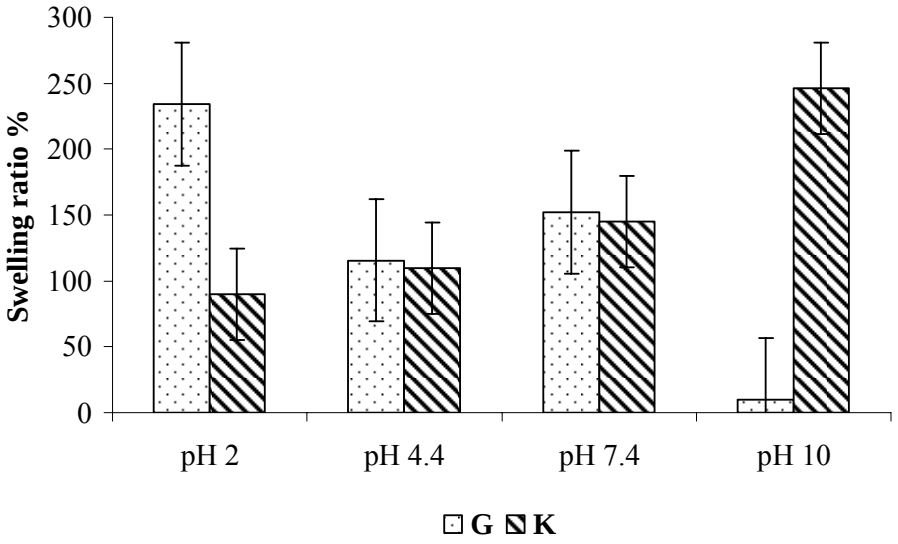
The swelling ratio determines the water content held by hydrogel. At a high pH (i.e. pH 10) due to the presence of cationic surface group the network becomes hydrophobic, hence having reduced capability to adsorb water. At neutral pH (i.e. pH 7.4) and even at lower pH (i.e. pH 4.4) the network becomes more hydrophilic and is capable of absorbing more water. This study demonstrates that linear PEG 1500-acrylate and gelatin not only strengthened the network structure of IPNs but make IPNs respond to pH change in an appreciable way.

Figure 22. Result from water swelling study of IPN composed of low PEGylated dendrimer G3.0 hydrogel after 24 hours of incubation i.e. at equilibrium swelling



G= G3-[PEG 12000-acrylate]$_3$ + linear PEG 1500-acrylate, **I**= G3-[PEG 12000-acrylate]$_3$ + Gelatin

Figure 23. Comparison of water swelling study of IPN composed of low PEGylated dendrimer G3.0 hydrogel and G3.5 dendrimer-PEG (1500) after 24 hours of incubation



G= G3-[PEG 12000-acrylate]$_3$ + linear PEG 1500-acrylate, **K=** G3.5-[PEG 1500-acrylate]$_{43}$

Figure 23 gives a comparison of water swelling behavior between full generation (G3.0) and half generation dendrimer (G3.5) based hydrogel. The half generation dendrimer has carboxyl group on the surface. It was observed that at low pH (i.e. pH 2), half generation dendrimer based hydrogel showed a lower swelling ratio (89.9 %) indicating less water absorption. It is assumed that the hydrogel network based on G3.5-[PEG 1500-acrylate]$_{43}$ becomes hydrophobic at pH 2, and less water is absorbed while, at pH 10 the network becomes hydrophilic and absorbs more water (i.e., the swelling ratio of 246% at pH 10). Thus % *increase* in swelling ratio for G3.5-[PEG 1500-acrylate]$_{43}$ from pH 2 to pH 10 is 95.73 %. G3.0-[PEG 12000-acrylate]$_3$ + linear PEG 1500-acrylate shows high swelling ratio (234.3%) at pH 2 and lower swelling ratio(10%) at

basic pH (i.e. pH 10). Thus % *decrease* in swelling ratio for G3.0-[PEG 12000-acrylate]$_3$ + linear PEG 1500-acrylate from pH 2 to pH 10 is 173.64 %. However when G3.5-[PEG 1500-acrylate]$_{43}$ hydrogels were compared with low PEGylated dendrimer G3.0-[PEG 12000-acrylate]$_3$, the difference between swelling ratio at high and low pH was less pronounced because G3.5-based hydrogels prepared had 43 out of 64 carboxyl groups of G3.5 dendrimer that were conjugated with PEG as compared to 3 out of 32 amine groups conjugated with PEG for low PEGylated G3.0 dendrimer. The higher degree of PEGylation reduces the number of exposed surface groups that are responsible for pH sensitivity of the network.

4.5 Degradation studies

It was observed that hydrogel degraded during incubation in the medium. Degradation degree at different pHs was quantified. The weight of salt entrapped within the matrix was neglected in the calculation of degradation ratio as the amount of salt was minimal, only accounting for 0.4 wt % of the overall weight of the hydrogel.

Figure 24. Degradation of G3-[PEG 12000-acrylate] $_{28}$ after 24 hours of incubation

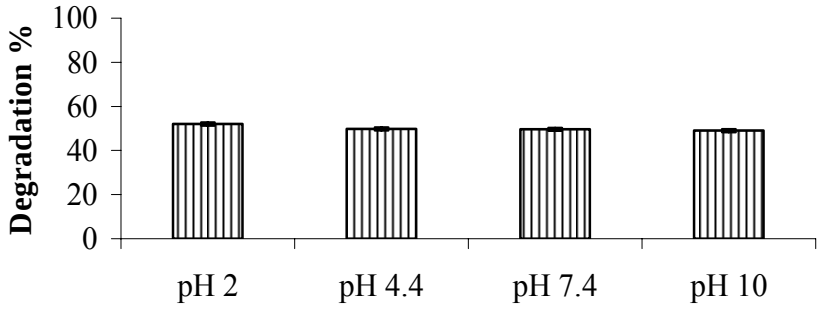


Figure 25. Degradation of IPN composed of G3-[PEG 12000-acrylate]$_3$ + linear PEG 1500-acrylate after 24 hours of incubation

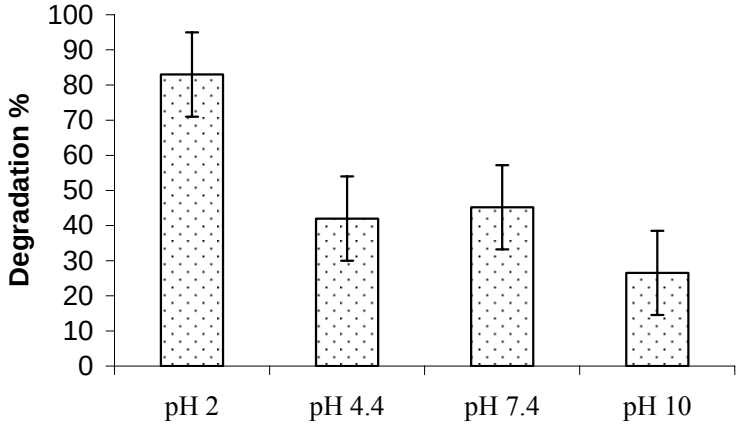
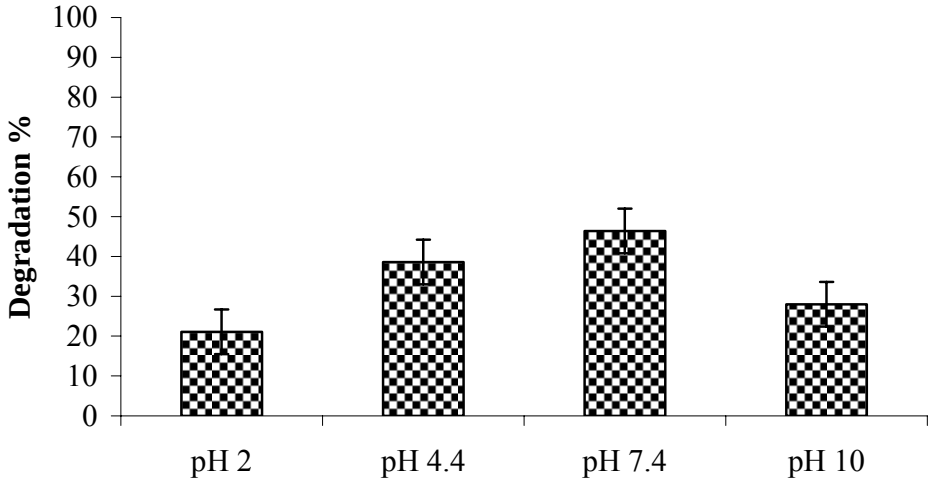


Figure 26. Degradation of IPN composed of G3-[PEG 12000-acrylate] $_3$ + gelatin after 24 hours of incubation



As seen in figure 25- 26 the degradation % for G3-[PEG 12000-acrylate]$_3$ + linear PEG 1500-acrylate at acidic pH was around 80 % while at basic pH was 30 % while G3-[PEG 12000-acrylate]$_3$ + gelatin showed very low degradation% as compared to G3-[PEG 12000-acrylate]$_3$ + linear PEG 1500-acrylate. The degradation % was around 30 % at pH 2, 40 % at pH 4.4 and 50 % at pH 7.4, and 28 % at pH 10. As illustrated

form these 2 figures the trend of degradation at different pH varies between G3-[PEG 12000-acrylate]$_3$ + linear PEG 1500-acrylate.and 3-[PEG 12000-acrylate]$_3$ + gelatin. This variation may be attributed by gelatin which might confer some amount of pH sensitivity.

Figure 27. Degradation of half generation dendrimer (G3.5-[PEG 1500-acrylate]$_{43}$) after 24 hours of incubation

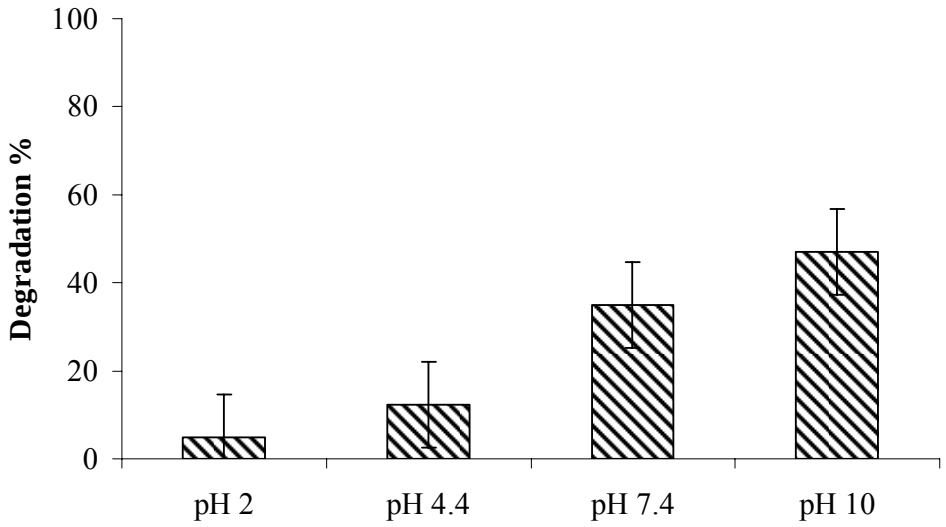
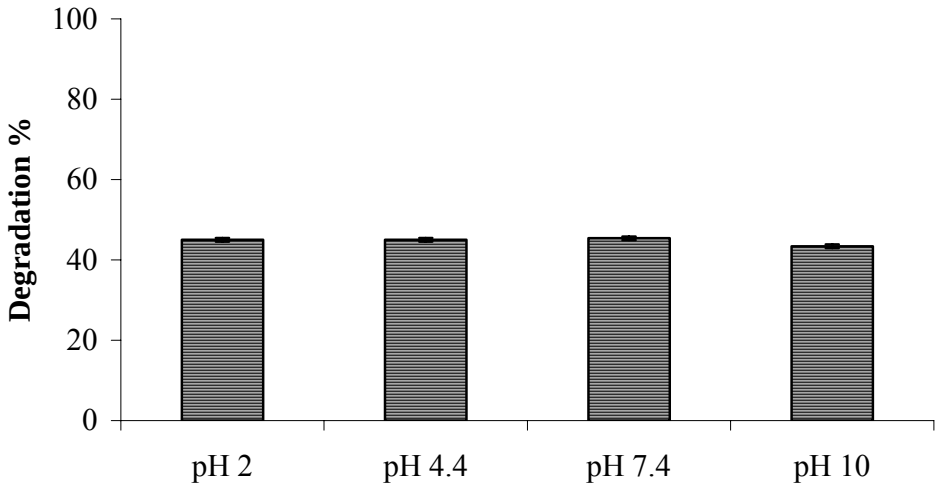


Figure 28. Degradation of Linear PEG 1500-acrylate after 24 hours of incubation

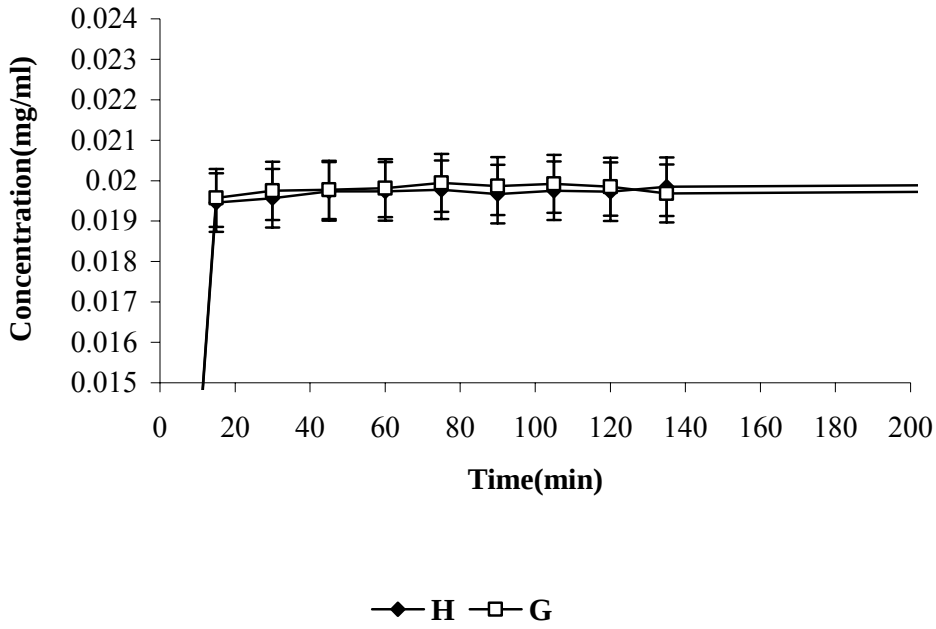


The degradation results were consistent with water swelling results and further confirmed the pH sensitivity of IPNs. From figures 25-27 it can be observed that the degradation rate of full generation dendrimer-based IPN was more pronounced at acidic pH (i.e. 80% at pH 2) than at basic pH 10 (26.4%). The degradation rate was reduced by 68.2 %. In contrast, half generation dendrimer-based hydrogel had a higher degradation ratio (47%) at pH 10 and a very low degradation ratio 4% at pH 2. The decrease from pH 10 to 2 caused decrease in the degradation ratio by 91 %. It was observed that the degradation ratio of linear PEG-acrylate hydrogels remained constant at 46 % at different pHs. It was also observed that fully PEGylated G3.0 dendrimer have a constant degradation ratio of 49% at different pHs. This was due to high degree of PEGylation, which significantly reduced the number of available amine groups that are responsible for pH sensitivity.

4.6 Drug release kinetics

Typically, researchers predict the release of active drugs as a function of time, using simple or sophisticated mathematical models. Diffusion controlled drug release is the most widely used model. Cyclosporine A, which is sparingly water soluble, was used as a model drug to understand the release kinetics. When the hydrogel is placed in medium, the network absorbs water, hence causing swelling of the network and subsequent drug release. In our study, hydrogels were placed in dialysis bag and positioned in a beaker filled with medium. The amount of drug being released was measured as a function of time

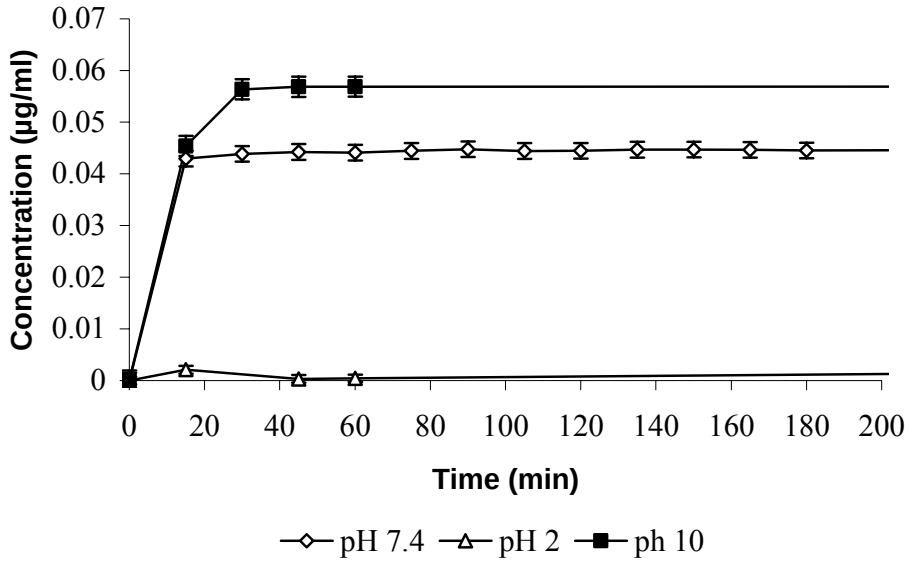
Figure 29. Release of cyclosporine A from IPN sample G and H in 100 ml of PBS buffer at pH 7.4 (drug encapsulation and IPN preparation was based on ethanol)



G= G3-[PEG 12000-acrylate]$_3$ + linear PEG 1500-acrylate, **H=** Linear PEG 1500-acrylate

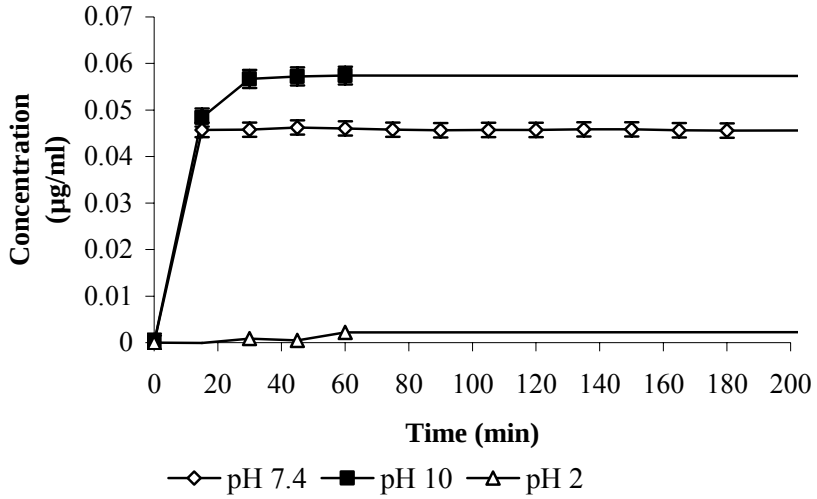
Figure 29 demonstrates release of Cyclosporine A from IPN sample G (G3.0-[PEG 12000-acrylate]$_3$)+linear PEG 1500-acrylate) hydrogels at pH 7.4 along with control linear PEG 1500-acrylate hydrogel which were formed using ethanol. The maximum amount of drug released was 2mg. The maximum concentration was quickly reached within a short time period (i.e. 40-50 min).

Figure 30. Release of cyclosporine A from linear PEG 1500-acrylate hydrogel at different pHs in 100 ml of medium



According to Figure 30, the maximum amount of drug released was 5.77 µg at pH 10, 1.26 µg at pH 2, and 4.66 µg at pH 7.4. All the hydrogel samples loaded with cyclosporine A were prepared under the same condition. Therefore, the actual amount of drug loaded into each sample was kept at the same level. However, we see a variation in drug release kinetics at different pHs. The drug has amine groups which may play a role in exhibiting pH sensitivity even though the network itself, which is made of linear PEG, does not. At high pH, the drug becomes more hydrophobic and hence cannot be retained by the network and repels the drug and higher release is observed. However at low pH the drug can be retained more tightly and released slowly.

Figure 31. Release of cyclosporine A from full generation dendrimer-based IPN sample at different pH in 100 ml of medium



It was expected that full generation dendrimer-based hydrogel could release drug more slowly at pH 10 than at pH 2, which, however, was not confirmed with our observation. The maximum amount of drug released was 5.77 µg at pH 10, 1.31 µg at pH 2, and 4.67 µg at pH 7.4, which were similar in value to those observed with the control. It was believed that the model drug was not appropriate enough as it presents itself with some amount of pH sensitivity (Figure 32) which could impact its release. Hence the pH sensitivity of the network can not be demonstrated very well as seen with swelling and degradation studies.

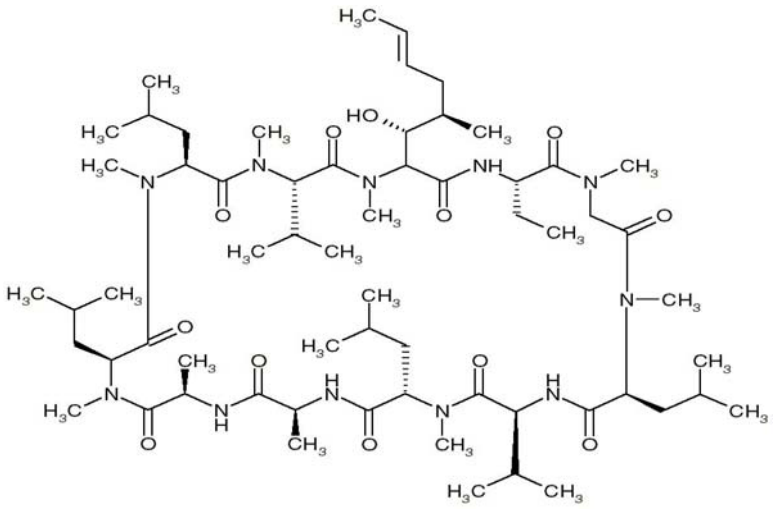
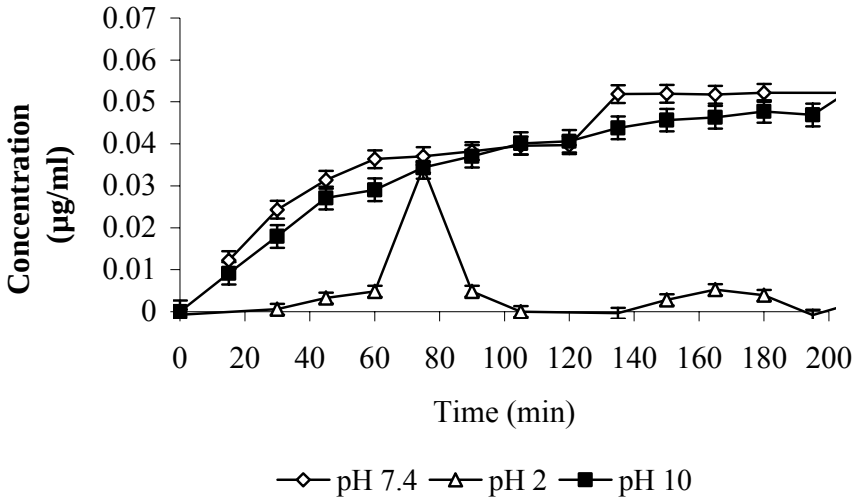


Figure 32 Structure of cyclosporine A

Figure 33. Release of cyclosporine A from half generation based dendrimer (G3.5-[PEG 1500-acrylate] $_{43}$) hydrogel at different pHs in 100 ml of medium



The release results (Figure 33) were well consistent with what we expected for half generation dendrimer-based hydrogel. It was observed that the drug release had a high rate at pH 7.4 and pH 10) while a lower rate at pH 2.

As both pH 7.4 and pH 10 were well above the pKa of carboxylate group on the dendrimer surface, the hydrogel had similar hydrophilicity at pH 7.4 and pH 10, resulting similar release rates for pH 7.4 and pH 10. Because the hydrogel became hydrophobic at pH 2, the release of drug was slowed down due to network shrinking.

4.7 Cell cytotoxicity and cell internalization studies

We initially evaluated cytotoxicity and internalization of the synthesized nanoparticle and nanomatrices by using RAW264 mouse macrophages. Uncrosslinked and crosslinked dendrimer-PEG displayed dose-dependent cytotoxicity; however, they had a negligible toxic effect on the cells at concentrations of 0.2 μM or below. Trypan Blue assay was performed to evaluate the toxic effect of the polymer before and after crosslinking. For *in vivo* application of hydrogel, along with the drug encapsulation and release ability, we should ensure that these hydrogels do not cause toxic effects on the cell and cause cell death. The cell viability was evaluated in vitro using RAW264 mouse macrophages cell lines. It was observed that lower the concentration better the cell viability for an exposure of 48 hours. It is important that the photoinitiator is biocompatible as it may leech out of the hydrogel network and cause adverse reaction. There was not much difference in the cell viability at same concentration before and after crosslinking with photoinitiator or an exposure period of 48 hours.. Thus it can be said that photoinitiator is compatible with the cells for an exposure period of 48 hours. A concentration of 0.2 μM showed 100 % cell viability for 48 hours of exposure. It is important to evaluate the behavior and interaction of cells when exposed to the polymer or when hydrogel is incorporated within the body. Cells were incubated in the polymer before forming hydrogel and after hydrogel formation. This would help understand cell interaction with the polymer and its distribution with the hydrogel network. Higher concentration and longer incubation of uncrosslinked dendrimer-PEG with macrophages resulted in stronger particle internalization. Further, the occurrence of the internalization of uncrosslinked dendrimer-PEG was observed within 15 minutes. In

contrast, internalization of nanomatrices by macrophages was significantly low and only observed after 60-min incubation**.**

Figure 33. Cytotoxicity of FITC-G3.0-[PEG 12000-acrylate] $_{28}$ before crosslinking for exposure of 48 hours

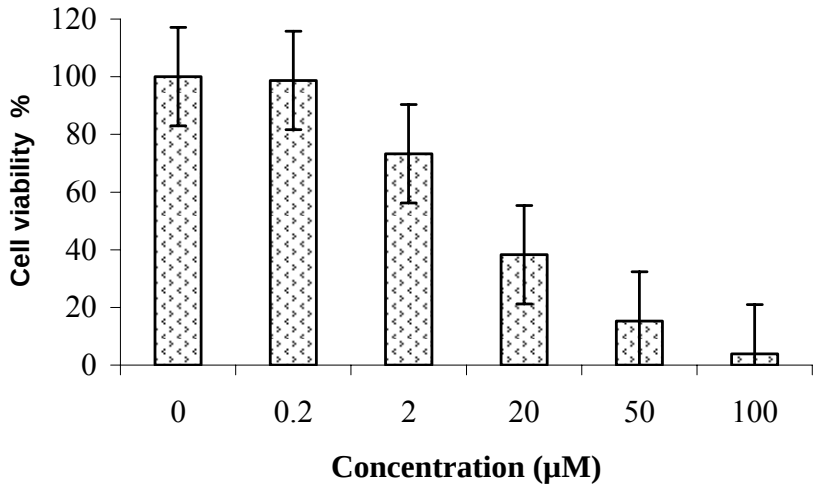
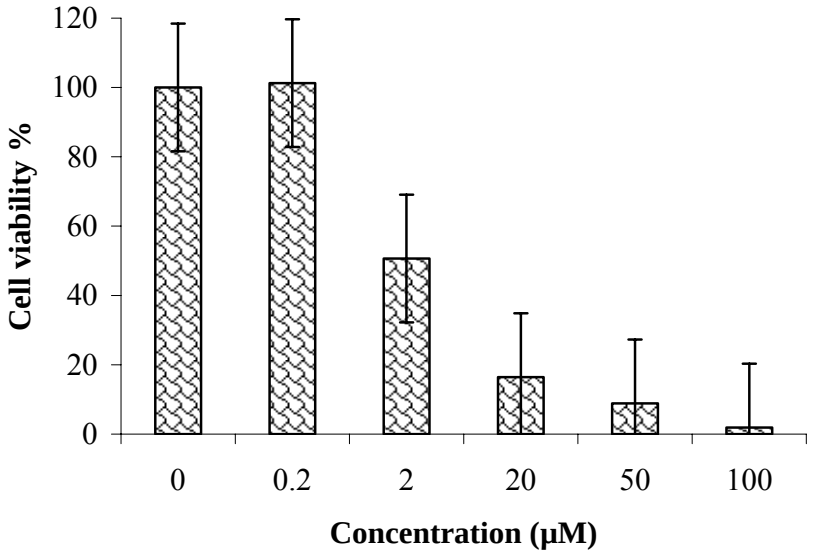


Figure 35. Cytotoxicity of FITC-G3.0-[PEG 12000-acrylate]$_{28}$ hydrogel for exposure of 48 hours



4.8 Conclusions

Our work demonstrated that polyionic hydrogels can be formed using full and half generation dendrimers. PEG lengths affect the hydrogel formation in terms of structure stability and gelation process. Three different PEGs i.e.1500, 6000, and 12000 were covalently conjugated to dendrimer. PEG with MW of 12000 yielded better hydrogel formations. The hydrogel formation also varied with the generation of dendrimer used. Half generation dendrimer could form better hydrogel with PEG length of 1500 as compared to full generation dendrimer conjugated to PEG 1500. The swelling ratio of full generation dendrimer G3.0 with a high degree of PEGylation showed no variation under different pH conditions as they did not possess enough surface groups, while full generation dendrimer with a low degree of PEGylation, demonstrated pH sensitivity in terms of swelling and degradation behavior. Also attempts were made to minimize the

UV exposure for hydrogel formation so that it can be used for *in situ* hydrogel formation. We demonstrated that hydrogels can be formed by day light exposure over a period a time and thereby UV radiation can be avoided, which may be needed in some cases. The hydrogels formed from full generation dendrimer can be implemented for ocular drug delivery. Since these hydrogels present with cationic charges, they can help to have longer retention on the anionic cornea through ionic interactions. This would help increase compliance and promote efficient drug delivery. Half generation dendrimer-based hydrogels can be used for oral drug delivery as they can react to pH gradient. Half generation dendrimer-based hydrogels have maximum swelling ratio at basic pH and hence the drug can be diffused out while at acidic pH drug is trapped within the hydrogel network. Drug release kinetics demonstrated limited pH sensitivity as compared with swelling and degradation studies. A new model drug, pyrene, will be used for drug release studies by excluding the pH effect on drug itself.

CHAPTER 5 Summary and Future Work

5 Summary and Future Work

5.1 Summary

With advances in the field of biomaterials, three dimensional crosslinked matrices hydrogels have become one of the most widely researched systems for drug delivery. These networks can be tailored to deliver drug efficiently and more effectively. Dendrimers have been extensively used for drug delivery. However, the research developing dendrimer-base hydrogel is limited. Dendrimers present themselves with surface charges and this property can be utilized for developing polyionic hydrogels. Polyionic hydrogels were formed from full and half generation dendrimer. These hydrogels were characterized to quantify their physical morphology, understanding swelling behavior, and drug release kinetics for a range of pH. The study was accompanied with PEG hydrogels as control.

It was seen that realization of hydrogel formation and swelling behavior varied with different molecular weight of PEG conjugated to dendrimer. Higher PEG length resulted in more stable hydrogel formation and higher swelling ratio. The hydrogel network developed in this project is highly adaptable. It can be engineered for various applications. As the dendrimer network has inner hydrophobic cores included, both hydrophobic and hydrophilic drugs can be loaded. This network can be either

polyanionic or polycationic. Till date research on dendrimer based hydrogels[43, 53, 60] have been limited in terms of exhibiting pH sensitivity. Hence, the new dendrimer-based polyionic hydrogel network developed in this study will find a wide range of applications.

5.2 Future work

This study attempted to characterize physical properties, swelling kinetics and drug release kinetics of dendrimer based hydrogels for biomedical applications. Presently only swelling, degradation studies, and drug kinetics were done at room temperature and different pH conditions. Future work should involve expansion of this study at different temperature as well. One of the potential applications of hydrogel would be ocular drug delivery. Ocular drug delivery can be targeted as these hydrogels have positive charges, hence help adhere to cornea possessing negative charges. This mechanism can help to increase the residence time of hydrogel in the eye, thereby delivering right amount of drug without repeated dosing. We have studied the release kinetics of drug Cyclosporine A. The drug presented with some difficulties. The drug itself can present with some pH sensitivity. Hence a better model drug such as pyrene could be used for drug release kinetics. This procedure had limitations as the hydrogel structure was not stable. Hence when the network breaks down, though the disintegrated network can be held within the dialysis bag, the drug diffuses out thus interfering with the results of release mechanism. Hence the method to be used for experimenting release mechanism shall be optimized for obtaining accurate results. Future work should

involve more appropriate ways of estimating release of drug from hydrogel such as using mathematical models. It was observed that these hydrogel demonstrated some degree of adhesiveness, thus testing the tissue adhesive and mechanical properties will help to determine its use as a sealant for wound closure of tissue and also as mucoadhesive drug delivery application. Future work should involve mechanical testing either by using texture analyzer or other similar instruments which would help measure the viscosity of these hydrogel.

As emphasized previously, the hydrogel should be biocompatible in order to be used in biomedical applications. Hydrogels synthesized in the present work were tested in vitro, which provides sufficient preliminary evidence on the biocompatibility of the hydrogels. The cell line used was RAW264 mouse macrophages which is not universally used for testing cytotoxicity. The cytoxicity will be evaluated in a more rigorous way using L929 mouse fibroblasts, a standard cell line for cytotoxicity evaluation. However, in vivo tests are more important as they provide a better image of the biocompatibility nature of a biomaterial. Thus as a suggestion for future work, *in vivo* studies involving clinical trials on the hydrogels prepared in the present work would provide a better understanding of their biocompatibility. Furthermore, *in vivo* studies will confirm the findings from this work on the suitability of these materials for biomedical use.

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Literature Cited

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VITA

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