PHOTOFUNCTIONALIZABLE HYDROGELS VIA ORTHOGONALLY CLICKABLE DENDRONS

by

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To my family

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ABSTRACT

PHOTOFUNCTIONALIZABLE HYDROGELS VIA ORTHOGONALLY CLICKABLE DENDRONS

Hydrogels are three-dimensional, cross linked hydrophilic polymeric materials that have attracted much attention due to their key role in the development of drug delivery systems, biomolecular sensors and scaffolds for tissue engineering. Traditional synthesis of these materials has generally relied on photopolymerization of water soluble monomers. These radical based cross linking method leads to the formation of non-uniform heterogeneous gels due to the uncontrolled mechanism of the reaction. Furthermore, such hydrogels do not have a well-defined architecture and shows poor mechanical stability. Highly efficient ‘Click chemistry’ is utilized in synthesizing homogeneous and stable hydrogel networks. The set of chemical reactions that are classified as ‘click’ reactions have high yields under mild conditions, do not generate offensive by-products and have high selectivity. Photolitically functionazible PEG based hydrogels are synthesized from orthogonally functionalizable biodegradable dendrons containing alkene unit at their focal point and multiple alkyne groups at the periphery. Doubly ‘clicked’ hydrogels are first synthesized via Huisgen type [3+2] click cycloaddition and then photo patterned with thiol-ene click addition. Clicked hydrogels comprised of a polyethylene glycol based bioinert matrix that are further functionalized with dye molecules by appending thiol based ligands with positional control of the thiol-ene click chemistry. The efficiency of the Huisgen click reaction in various monomer/cross linker ratios is investigated. Photofunctionalization process is utilized by seeding the gels with florescent dye molecules. Photolithography technique is used in controlled patterning of the hydrogels under irradiation. Bioimmobilization on the hydrogel surface is demonstrated by biotin mediated attachment of FITC-Streptavidin.

ÖZET

ORTOGONAL DENDRONLARDAN FOTO-DUYARLI FONKSİYONEL HİDROJEL SENTEZİ

Hidrojeller üç boyutlu, hidrofilik, çapraz bağlı materyaller olarak; ilaç salınım sistemleri, biyomoleküler sensörler ve doku mühendisliği için ortamlarda kullanılma özelliklerinden dolayı çok ilgi çekmektedir. Bu materyallerin geleneksel sentezi suda çözünebilen polimerlerin foto-polimerizasyonu ile gerçekleşmekteydi ancak bu metod kontrolsüz gerçekleştiğinden heterojen yapıda bir hidrojel meydana getiriyordu. Oluşan hidrojellerin mekanik özellikleri zayıftı ve iyi tanımlanmış yapıları yoktu. Yüksek verimli klik reaksiyonu ile homojen ve stabil hidrojeller sentezlenebilmektedir. Klik reaksiyonu olarak tanımlanan reaksiyon kümesinin ortak özellikleri; hafif koşullarda yüksek verim, reaktif yan ürün oluşmaması ve yüksek seçicilikte olmasıdır. Çekirdeğinde alkin grup içeren ve yan dallarında alkanlar bulunan, ortogonal olarak fonksiyonlaştırılabilen biyo-bozunur dendronlardan foto duyarlı fonksiyonel hidrojeller sentezlenmiştir. Çift klik yöntemiyle sentezlenen hidrojeller önce Huisgen cinsi [3+2] siklokatılım reaksiyonu ile oluşturulmuş daha sonra tiol-alkene klik reaksiyonu ile foto-modelleme yapılmıştır. Kliklendirilmiş hidrojeller Polietilen Glikol içeren bioinert bir matrikse sahiplerdir ve tiol içeren boya molekülleri ile tiol-alkene pozisyon kontrolü sağlayarak tekrardan fonksiyonelleştirilebilirler. Klik reaksiyonun verimi değişik monomer/çapraz bağlayıcı oranları kullanılarak test edilmiştir. Foto-duyarlı fonksiyonelleştirme jellere boya molekülleri takılarak gösterilmiştir. Fotolitografi tekniği jel yüzeyine kontrollü modelleme ile uygulanmıştır. Hidrojel üzerinde biyo-immobilizasyon Streptavidin enzimi ile gösterilmiştir.

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LIST OF SYMBOLS

G	Generation
J	Coupling constant
υ	Frequency

LIST OF ACRONYMS / ABBREVIATIONS

$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Dichloromethane
DMAP	4-Dimethylaminopyridine
EtOAc	Ethyl Acetate
FITC	Fluorescein isothiocyanate
FT-IR	Fourier Transform Infrared
GPC	Gel Permeation Chromatography
MeOH	Methanol
MHz	Mega hertz
NMR	Nuclear Magnetic Resonance
$NaHSO_4$	Sodium hydrogen sulfate
Na_2CO_3	Sodium hydrogen carbonate
PDMS	Polydimethylsiloxane
PEG	Poly (ethylene glycol)
TEA	Triethylamine
TEM	Transmission Electron Microscopy
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
UV	Ultraviole

1. INTRODUCTION

1.1. Hydrogels

Hydrogels are cross linked systems composed of mainly water soluble polymers that are water absorbent, preferably antibiofouling and non-immunogenic.(Figure 1.1)Commonly used polymeric backbones are poly (ethylene glycol) (PEG), Chitosan, poly vinyl alcohol (PVA), poly lactide(PLA), poly (N-isopropylacrylamide) (PNIPAm) and many more water soluble polymeric systems are used. [1-7] PEG is the most attractive hydrogel matrix for being non-charged, bioinert and having excellent swelling ability.

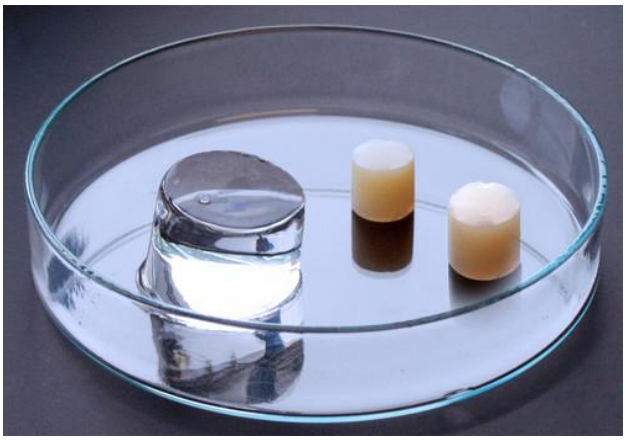

Figure 1.1. Representative Hydrogel Images [8].

Hydrogels are classified into 2 groups according to their cross linking characteristics. Physically cross linked hydrogels are composed of Hydrogen bonding or self-assembly of hydrophilic/hydrophobic groups. They tend to be reversible upon changing the temperature, pH or another parameter in the media by breaking the reversible bonds. Although most hydrogels in literature are composed of polymeric materials Xing and co-workers reported an example that does not use the polymeric supports in the hydrogel. By incorporating pyrene units for support and the pi-pi stacking between the pyrene units crosslink the network and form a hydrogel (Figure 1.2) [7].

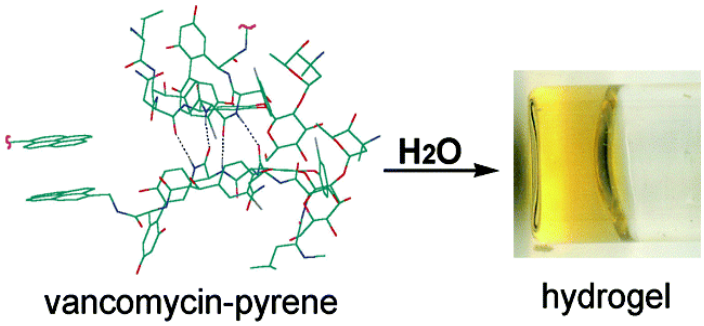

Figure 1.2. Physical Hydrogel from an antiobiotic and pyrene units [9].

In order to construct a stable network, chemical cross linking of polymers is achieved via covalent bond formation. They generally form non-reversible and more rigid hydrogels.

Examples for chemically cross linked hydrogel networks with the most commonly used mechanisms can be given as; azide functionalized PVA's clicked with alkyne appended PVA's [10], polymerization of peptides with ATRP [11], crosslinking with Diels-Alder reaction between PEG-bismaleimide and furan containing polymers [12], PEG-tetrathiols clicked with bisacrylates [13] to give hydrogel networksand photo cross inking of acrylates in the presence of a photo initiator [14]. In deed by changing the type of the polymer or reaction method or reaction conditions, we can tune the properties of hydrogels such as their swelling ability, cross link density, biodegradability, biocompatibility and functionality.

As a consequence of this adjustable features and modifications, today's hydrogels are widely used in medical applications for example targeted drug delivery, cell growth and artificial tissue engineering, diagnostics, wound healing coatings, biological adhesions, and most probable candidate of transplantation.

With the expanding utilization of PEG based polymers in hydrogel networks, screening of biological events have become easier. The natural polymers interfere with the mechanisms and creates an inconvenient media. PEG is bioinert therefore no interfere occurs with the ongoing reactions [15]. Also for the cell culturing and screening applications, reaction media plays a key role. Optimum biological conditions should be satisfied including the natural humidity of the media. Not only for screening but also for tissue engineering applications, hydrogels should retain water in certain limits [16]. PEG is

an excellent candidate for its high water retaining ability since it can easily form H bonding with water and it has a highly water soluble nature.

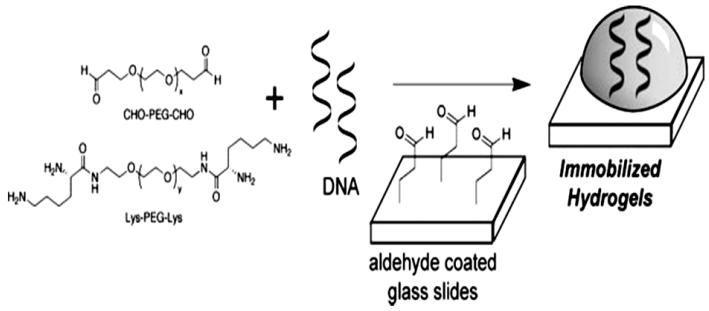

Figure 1.3. Hydrogels for screening events [15].

1.2. Click Reaction for Hydrogel Formation

Click chemistry is a term introduced by K. B. Sharpless and colleagues defined as the bond formation with high efficiency and selectivity [17]. First click reaction were to mean efficient carbon-heteroatom bond formation. There are couple of reactions, which can be defined as a click reaction. 2+4 Diels Alder cycloaddition between an ene and a diene, Michael addition of thiols to acrylates and Cu catalysed Huisgen type click reaction (CuAAC). Most recently Cu free click chemistry has been introduced by Anseth and colleagues and used in situ. [18] There are criteria that have been set for one reaction to be defined as 'click chemistry'. It has to be highly efficient, modular, wide in scope and generate inoffensive by-products [17]. Click chemistry has been used to construct a wide range of materials including small molecules, polymers, dendrimers and hydrogels. Hawker group synthesized dendrimers with CuAAC [19] and thiol-ene coupling [20] showing the excellent efficiency click chemistry. Diels Alder cycloaddition, (DA) and retro Diels Alder reaction (rDA) has been used extensively for the ability of reversing the reaction via temperature [21].

Hydrogel chemistry was lacking homogeneity and control over the reaction back in 1960's when first hydrogel-like material was synthesized by Wichterle using HEMA [22].

Control over reaction gives the advantage of tuning the network formation in terms of cross linking density, functional group density, swellability of the hydrogel. Reaction control in network formation is achieved via step-growth polymerization meaning an orderly growing mechanism. Opposite case is the photo cross linking of acrylates under UV irradiation with suitable initiators where intramolecular cross linking may dominate the reaction in some conditions resulting in an unwanted network. Click chemistry being a step-growth mechanism creates a radical and initiates the polymerization by grabbing a proton from an electron rich centre. Propagation step is the growth of the polymer from the chain ends creating a radical in each step of growth. Since the reaction is only intermolecular and ordered the final network is homogeneous in terms of crosslink density and functional group density.

1.2.1 Huisgen Type Click Reaction

The most famous Click reaction is Huisgen [3+2] cycloaddition reaction. Azide-alkyne cycloaddition reaction was introduced by Rolf Huisgen in 1970 without using water and catalyst, azide and alkyne groups react at high temperature to produce a mixture of 1,4 and 1,5-disubstituted triazoles (Figure 1.4).

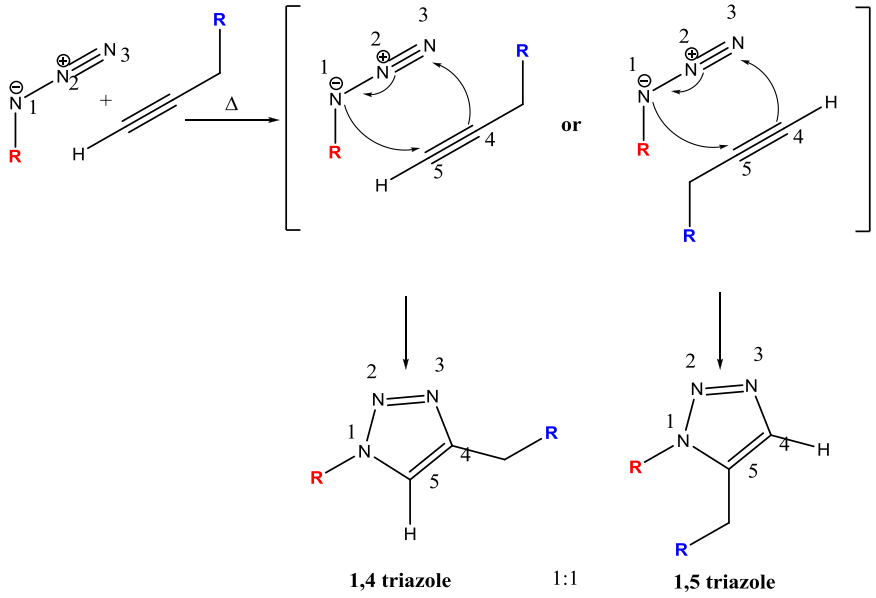

Figure 1.4. Huisgen type Click reaction.

In 2004 Sharpless and Mendal [17] introduced a Cu (I) catalyst in Huisgen reaction that the catalyst directs the region-specific result of the reaction and results in only 1,4-disubstituted triazole (Figure 1.5). This reaction is commonly referred as the copper (I)-catalyzed azide-alkyne cycloaddition reaction.

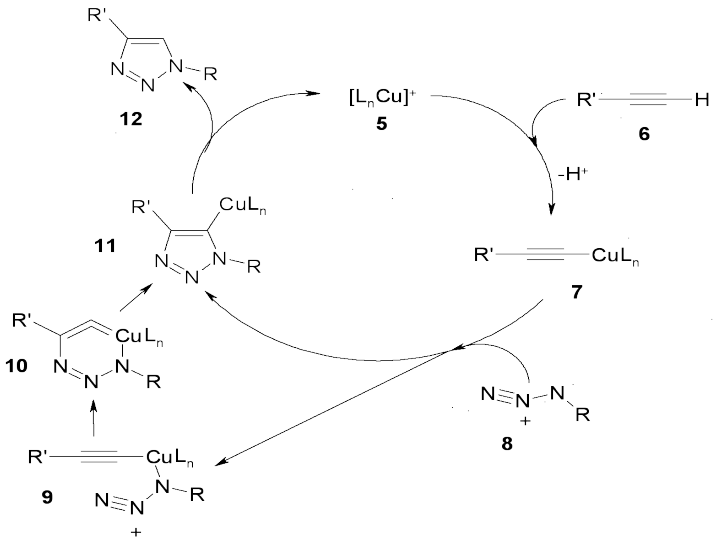

Figure 1.5. Mechanism of the Cu catalysed Click reaction introduced by Sharpless and coworkers [17].

The first group to synthesize a clicked hydrogel network is Hilborn et al. PVA's modified with carbamate linkages containing alkyne and azide pendant groups are clicked with Cu catalyzed Huisgen reaction [23]. Reaction conditions are tested in terms of functional group concentration, polymer components and catalyst concentration. The result shows that there is a minimum for the number of corresponding pendant groups to form hydrogel and there is a maximum where the gel fraction is not affected but swelling ability decreased as the crosslinking density increased. As the hydrophilicty of the components increased, swelling ability of the hydrogel increased. There is a minimum for the catalyst concentration where gel fraction drops down immediately due to the succumb of side reactions.Divalent polymers tend to make intramolecularcrosslink than intermolecular which result in lower gel fractions and lower elastic modulus. Results show the divalent PEG-diazides cross linking with PVA-alkynes yielding highly swollen but low modulus hydrogels.

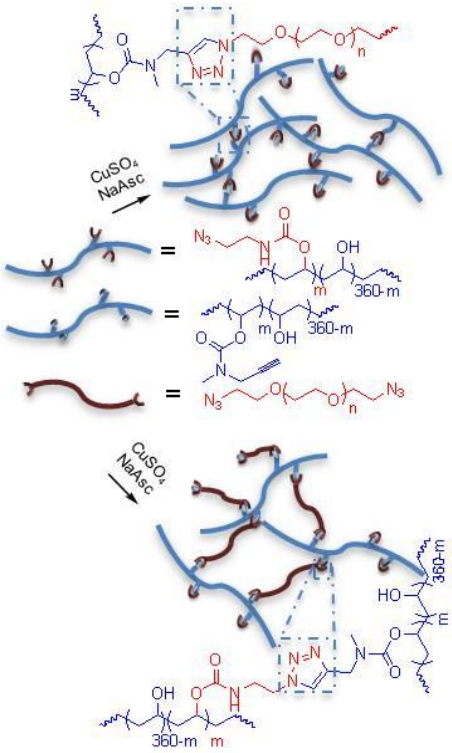

Figure 1.6. Huisgen type clicking PVA alkyne and azide polymers [23].

Hawker and coworkers reported an elegant use of the Cu-catalyzed Huisgen type ‘click’ reaction to obtain PEG-based hydrogels with controlled architecture and improved mechanical properties (Figure 1.7). [24] PEG was utilized in this work for its high biocompatibility and hydrophilicity. The hydrogel was obtained by the ‘click’ reaction between diacetylene functionalized linear PEG polymers and tetraazide functionalized tetraethylene glycol. High gel conversions (>95%) were observed at room temperature in less than 30 min, which could be further decreased to less than a minute using microwave irradiation. The mechanical properties of these hydrogels could be fine tuned by varying with the molecular weight of the polymer. More importantly, considerably better mechanical properties were observed for the hydrogels prepared via the ‘click’ method when compared to hydrogels prepared via free-radical photopolymerization of PEGdiacrylates. To probe the efficiency of the ‘click’ reaction in generating ideal model networks, analysis of residual azide and alkyne residues were performed. Azide and alkyne functionalized chromophores were appended to the residual reactive groups within the hydrogels. UV and florescence analysis showed that a maximum of 0.2% of unreacted

functional groups are present after the gel formation. This seminal contribution provides a clear demonstration of the ability of ‘click’ reactions to provide access to hydrogels that are molecularly well-defined and possess near-ideal network structures.

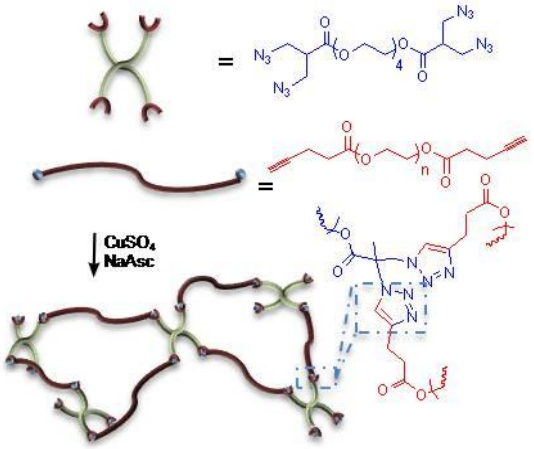

Figure 1.7. PEG based hydrogels via CuACC [24].

Enzymatically biodegradable hydrogel networks are attractive smart materials that can be utilized as drug delivery platforms to release entrapped bioactive materials upon exposure to specific cell-secreted and cell-activated enzymes. Recently, Hennick and Liskamp fabricated such hydrogels by incorporating an ezymatically cleavable peptide sequence into the PEG based hydrogel matrix [25]. The tripeptide sequence, D-Ala-Phe-Lys, a known selective substrate for the serine proteases trypsin and plasmin was utilized as a cleavable crosslinker unit to achieve enzyme-responsive degradation (Figure 1.8). Alkyne functionalized 4-arm and 8-arm PEG polymers were crosslinked with the enzyme sensitive bisazido tripeptide using the $CuSO_4$/NaAsc catalyst system. It was noted that hydrogels upon incubation with trypsin completely degraded in 40-80 h under physiological conditions. Surprisingly, these hydrogels were stable against plasmin even after incubation for 200 h. It was proposed that this could be due to either the large size of plasmin compared to trypsin that prevents its access into the hydrogels or the tripeptide unit upon attachment to the hydrogel becomes sterically inaccessible to the plasmin enzyme or its active site.

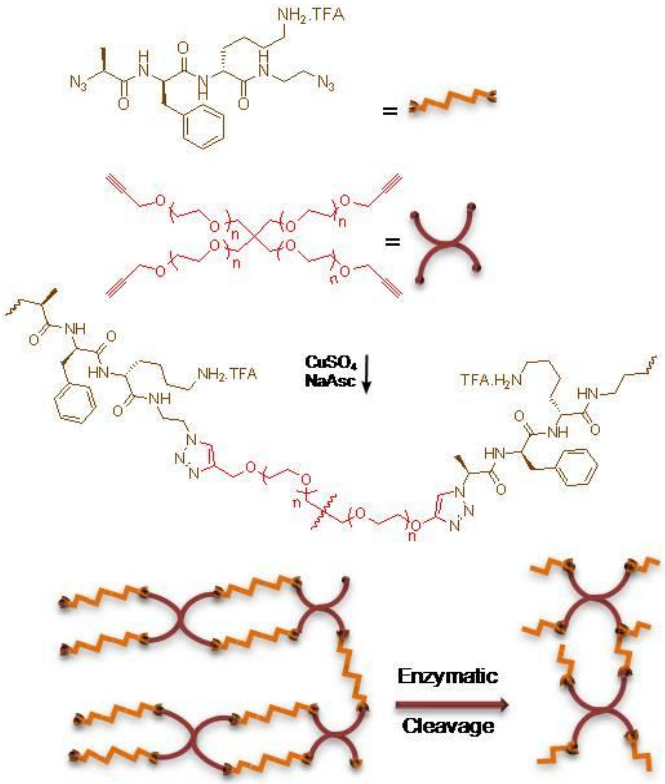

Figure 1.8. Biodegradable Hydrogel Network via Enzymatic Cleavage [25].

In a related approach, Kopecek and coworkers synthesized enzymatically degradable hydrogels by click reaction of 4-arm azido terminated PEG and alkyne terminated peptides [26]. The hydrogels degraded when incubated with the enzyme, papain. It was observed that hydrogels fabricated with longer PEG chains degraded much faster than the ones with shorter PEG chains.

1.3 Dendrons for Hydrogel Formation

Dendrimers are highly branched, unimolecular and globular structures and are classified as one of the polymer subgroups. They are composed of three components: a core, branching units and surface groups. (Figure 1.9)

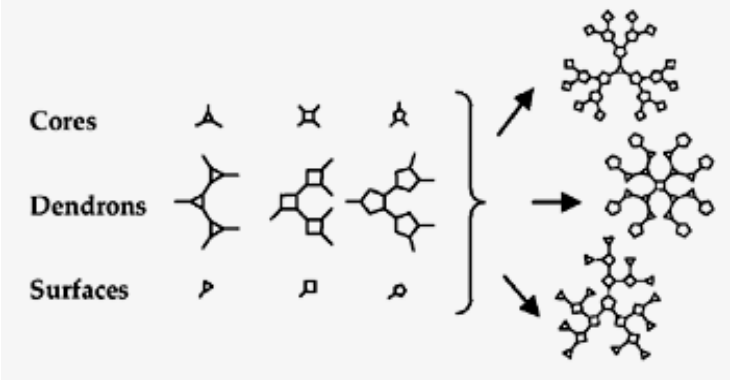

Figure 1.9. Representative synthesis of dendrimers.

Every component of the dendrimer can be used as a tool to attach molecules onto the structure. A core can be used as a linker or attached to a functional group. Branching units can be used according to the hydrophilic characteristics, to solubilize the whole structure or to carry a water soluble drug via non-covalent interactions. Internal cavities also play an important role as carrying agents through non-covalent interactions. Peripheral groups are the most popular part of the dendrimer. They provide functional groups in excess and exact amounts which are well-defined in the whole structure (Figure 1.10).

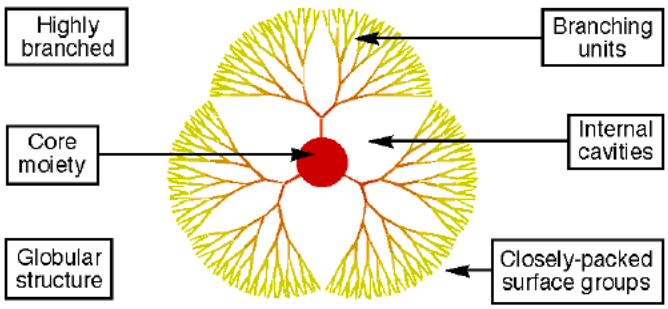

Figure 1.10. Parts of the dendrimer for post-modification.

Dendrons are a small part of the dendrimers which are composed of a focal point, side chains and peripheral units. They are not globular but have numerous functional groups according to their generation number. A generation number is a characteristic of a dendron which implies its number of functional groups. 2^n: n being the number of

generation is the number of functional groups on the dendron. The number of functional groups increases exponentially on the dendron.

Dendron can be synthesized by following one of the 2 methods: convergent or divergent. Convergent method start the synthesis from the branching units, in other words from the periphery to the core. Divergent method is the opposite way which starts the synthesis from the core and adding the branching units on top. (Figure 1.11)

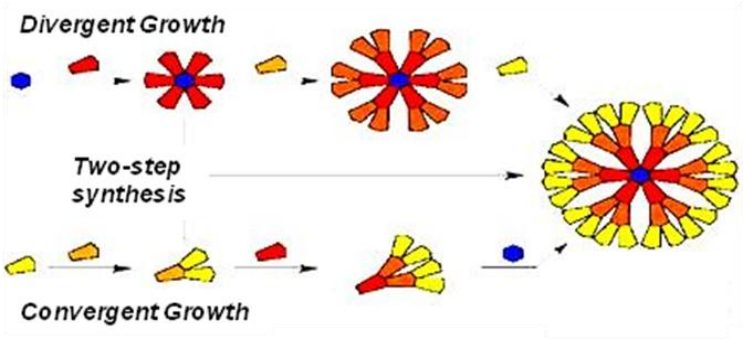

Figure 1.11. Convergent and Divergent Approach for dendrimer synthesis.

The main reason for the incorporation of dendrons into the polymeric networks is to enhance the functionalization ability of the whole system. There are numerous examples for the incorporation of dendrons into the polymer chains as side chains or end groups to increase the loading capacity or to define the place of the functional groups.

The advantages of dendritic macromers for hydrogel formation include high cross linking densities at low polymer concentration, varied physical properties of the macromer structure and low viscous aqueous solutions for injection in an in vivo state of irregular shape for subsequent cross linking to form a well-integrated polymer network.[26] Grinstaff and coworkers synthesized dendron-polymer conjugate based hydrogels for ocular treatment after the cataract surgery (Figure 1.12) [27]. The use of a hydrogel sealant as opposed to nylon sutures in cataract surgeries provides a facile method to safely and effectively seal the incision.

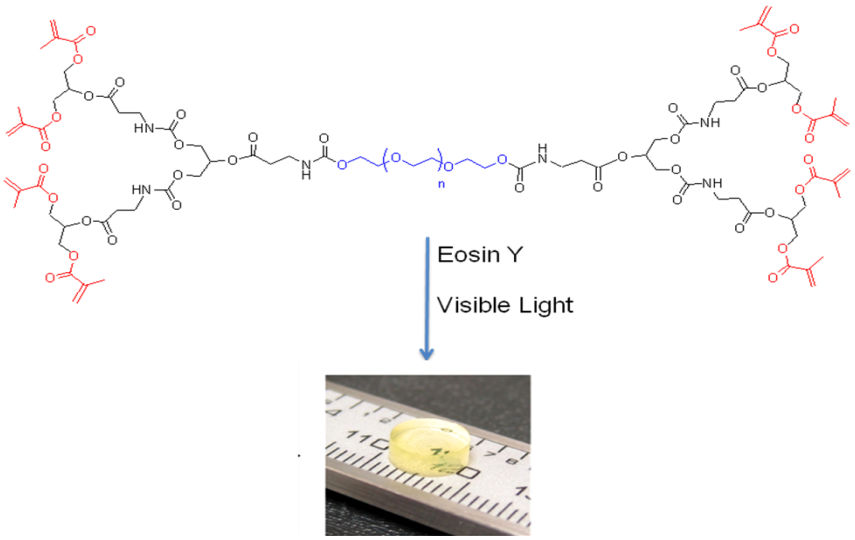

Figure 1.12. Hydrogels for ocular treatment under visible light [27].

Recently, efficient post functionalization of hydrogels in a controlled manner was demonstrated by Sanyal et al. on a reactive hydrogel fabricatedusing a dendron-polymer-dendron conjugate. [28] The synthetic design of hydrogel formation allowed control over the number of residual clickable alkyne groups in the hydrogel (Figure 1.13). Thus, precise control over the extent of covalent functionalizaton of these gels could be achieved.

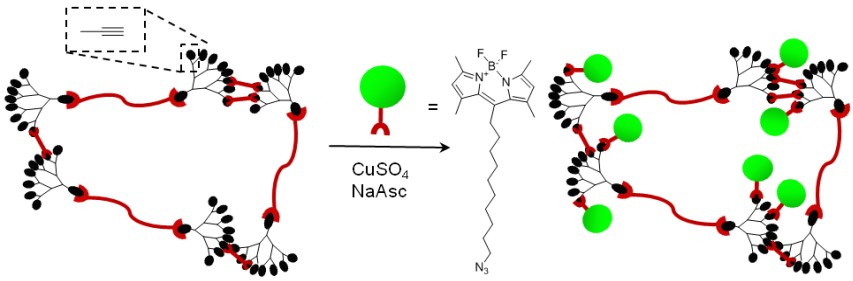

Figure 1.13. Hydrogel formation via CuAAC [28].

Hydrogels with varying content of residual alkyne groups were reacted with either azide containing BODIPY dye ora biotin ligand under standard Cu(I)-catalyzed Huisgen-type cycloaddition. Extent of immobilization of fluorescent dye or FITC-labeled

streptavidin onto these hydrogels was clearly proportional to the amount of reactive alkyne groups in the hydrogel.

1.4 Functionalization of the hydrogels

Hydrogels are used in numerous applications varying from gene delivery to molecular screening events. To be able to conduct such experiments appropriate modification of the hydrogel should be done. For example incorporating lysine units into the hydrogel structure is a better way than using PAMAM polymer for cell attachment. Bioimmobilization on the hydrogel surface is an attractive method not only to screen the enzyme activity on the hydrogel with attached living cells on it but also to perform different biological reactions on the same hydrogel platform without interfering with each other.

1.4.1 Thiol-ene Reaction for Functionalization

Functionalization of hydrogels becomes essential as the concept progresses. Post modification of the networks provide us with covalent loading of the gels with proteins, drug molecules etc. Also molecular modeling of the reactions in-situ can be screened through the small modifications on the hydrogel. The functionalization can be made sequentially by using different reactive but orthogonal groups on the same hydrogel. Also thiol-ene chemistry gives the advantage of selecting the reaction area by UV irradiation. (Figure 1.14) It is highly desirable that the post-functionalization of hydrogels can be carried out under a metal-catalyst free, mild condition, when modifications with sensitive biomolecules such asenzymes and protein therapeutics are intended.

Rapid and efficient characteristics rename the thiol additions to acrylates, vinyls via Michael addition or under UV, as click chemistry. At the end, free of side products is the most important feature of this reaction making it suitable for sensitive applications.

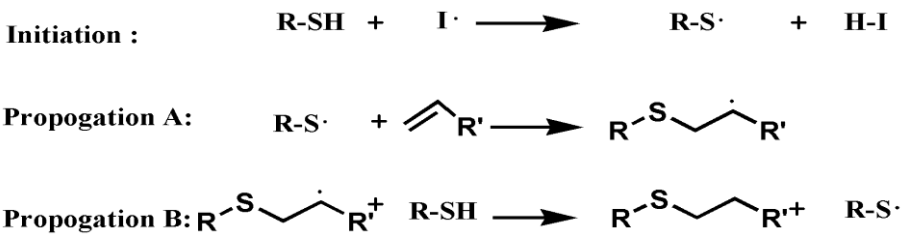

Figure 1.14. Thiol-ene Reaction Mechanism.

Thiol - ene chemistry does not require any complex reagents or harsh reaction conditions or solvents. UV irradiation dependency makes the reaction controllable [15]. Thiol containing molecules are readily absorbed physically by the gels and photo initiation completes the covalent attachment of the molecules to the directed regions. Covalent attachment to the polymer backbones having several thiol reactive groups have been shown to work efficiently in a short time.

Shoichet and coworkers reported an elegant example of spatially controlled 3-dimensional patterning within agarose hydrogels using a nucleophilic thiol-ene conjugation [29]. Agarose hydrogel was modified with a hydroxycoumarin sulfide derivative that can generate free thiol groups upon irradiation with either with UV-light or pulsed infrared laser. The chemically patterned thiol groups in the hydrogel matrix can be functionalized by maleimide containing molecules (Figure 1.15). Using multiphoton laser patterning, 3D patterns of thiol groups within the hydrogel matrix were generated. Sequential thiol generation and functionalization with different maleimide containing dyes allowed distinct spatially controlled multiple functionalizations. Although the report did not classify the functionalization as ‘click’ based strategy, the efficient capping of emanating thiols with maleimides under reagent free conditions demonstrates the ‘click’ nature of thiol-maleimide coupling reaction.

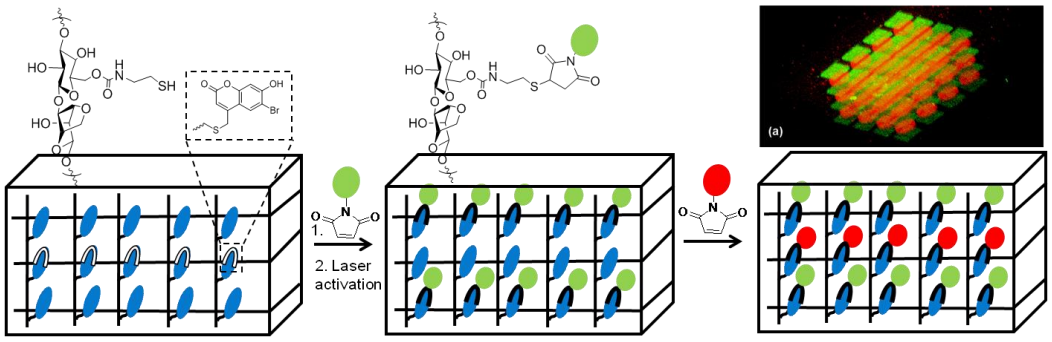

Figure 1.15. Functionalization of the hydrogel with pulsed infrared beam [29].

Anseth group used a 'PEG tide' hydrogel network for easy cell attachment [15].While the hydrogels themselves were synthesized via the Huisgen-type cycloaddition reactions, the photochemical thiol-ene reaction enables the patterning of these hydrogels with biological entities like thiol containing peptides in a spatially addressable manner with micrometer-scale resolution. Bio-immobilization on the hydrogel was shown by the photochemical thiol-ene reaction between the alkene units embedded in the hydrogel and fluorescently labeled thiol-containing peptides. The work reports the first example of fabrication of biochemical gradients of RGD containing peptides using photolithography on hydrogels with ideal-network structures.

The same group used catalyst free Huisgen type click reaction to construct the hydrogel network and functionalize via thiol-ene click, both reactions enabling the gelation application to be applied in situ, eliminating any side products or metal catalyst [30].

Control of the functionalization of cell loaded gels are done by using an enzyme sensitive fluorescently labeled peptide sequence. Functionalization area can be selected with photopatterning or two-photon technique. Enzyme activity occurring in the areas surrounded by cells manifests itself by fluorescing with higher intensity. This technique objects to give real-time information in the areas selected by the user.

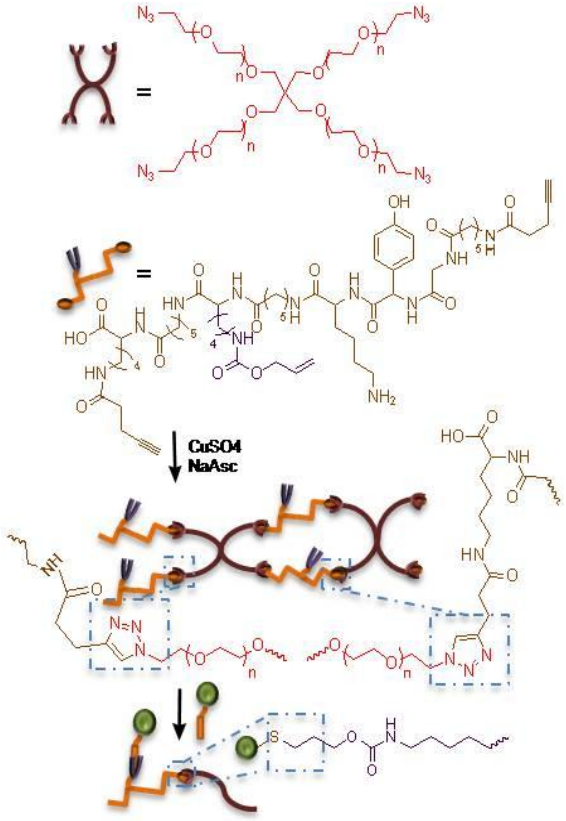

Figure 1.16. Bioimmobilization via thiol-ene functionalization [15].

Post functionalization of the hydrogel should be free of side products if used for in situ applications or immediate modification with sensitive biomaterials such as enzymes, protein therapeutics. Sanyal group achieved functionalization of the hydrogel via bioimmobilization of thiol containing biotin with Michael addition to free maleimide in the hydrogel network [1].PEG-based hydrogels containing different amount of maleimide groups were incubated with a thiol containing biotin derivative at ambient temperature to obtain hydrogels with different levels of biotinylation (Figure 1.17). The amount of FITC-streptavidin immobilization, as determined from fluorescence images of these gels was found to be proportional to the maleimide content in the parent hydrogels.

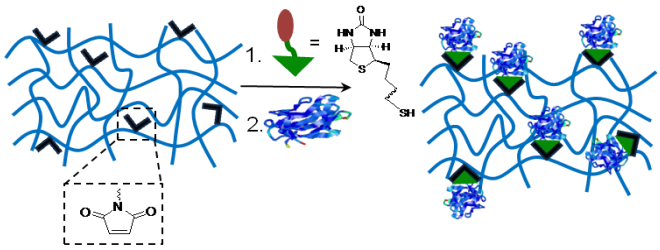

Figure 1.17. Post Functionalization via Thiol-ene [1].

1.4.2 Microcontact Printing for Hydrogel Patterning and Functionalization

Functionalization of hydrogels, especially at the micro and nanoscale, is increasingly gaining attention in the field of biology and medicine. Over the past few years, hydrogels applications have increased as functional components in biomedical micro and nanodevices.

Micro contact printing (or μCP) is a form of soft lithography that uses the relief patterns on a master Polydimethylsiloxane (PDMS) stamp to form patterns of self assembled of ink on the surface of a substrate through conformal contact. Its applications are wide ranging including microelectronics, surface chemistry and cell biology (Figure 1.18) [31].

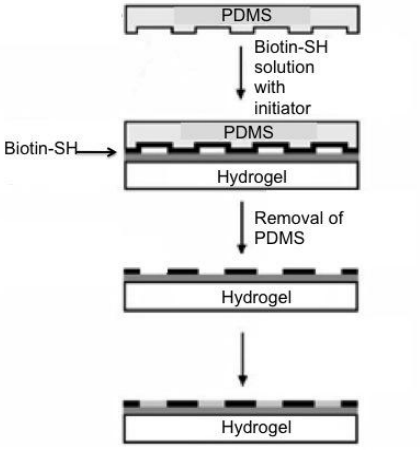

Figure 1.18. Illustration of Biotin-SH attachment on hydrogel via micro contact printing [31].

Microcontact printing (μCP) is a potentially biocompatible method for transfering biomolecules directly onto a large surface with a resolution of several micrometers. In micro-contact printing the affinity of the biological solute for the target surface has to be higher than its affinity for the stamp for the transfer to be efficient. It is the method of choice for printed features requiring high resolution [32]. The patterned feature size and resolution obtainable via micro-contact printing can be down to less than 100nm.

Chirra et al. used PDMS stamping to build nano scaled hydrogel patterns on the surface via atom transfer radical polymerization (ATRP). The development of a flexible platform for the controlled growth of hydrogel nanostructures on gold surfaces has been presented. Temperature responsive hydrogel systems were synthesized using ethylene glycol based crosslinkers of various molecular weights. Via micro contact printing two-dimensional control at the microscale is achieved. ATRP was used to successfully produce various hydrogel structures with controllable thickness [33]. The synthesis of several tunable intelligent hydrogel platforms at the nanometer scale holds significant promise for sensing and actuation in diagnostic and therapeutic applications, where hydrogel thin film actuators may manipulate the transportation, diffusion, entrapment, reaction, and detection of a multitude of biomolecules (Figure 1.19).

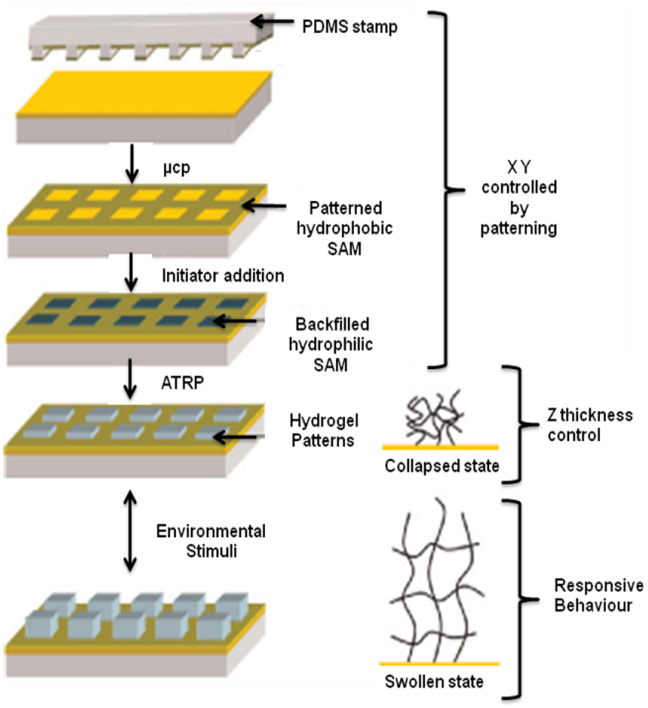

Figure 1.19. PDMS micro contact printing to form hydrogel patterns [33].

The soft protein lithography (SPL) technique of micro contact printing offers a way to control the patterning of molecules onto a surface. The molecule of interest is only patterned where the Polydimethylsiloxane (PDMS) stamp is in focal contact with the surface.

The novel acrylamide-based hydrogel that can be patterned with multiple biologically relevant molecules at micrometer-scale resolution is described. The hydrogel system can be used to provide precise control of the patterning of multiple biomolecules. By variation of the Streptavidin content of the hydrogel, protein density within patterns can be optimized. Furthermore, use of linkers with differing lengths between the biomolecules and the biotin moiety would permit tethering of ‘‘soluble’’ molecules with prescribed densities and patterns. [34] The ability to thus specifically patternhydrogel surfaces with a variety of molecules has major implications for the future design of patterned biomolecular devices (Figure 1.20).

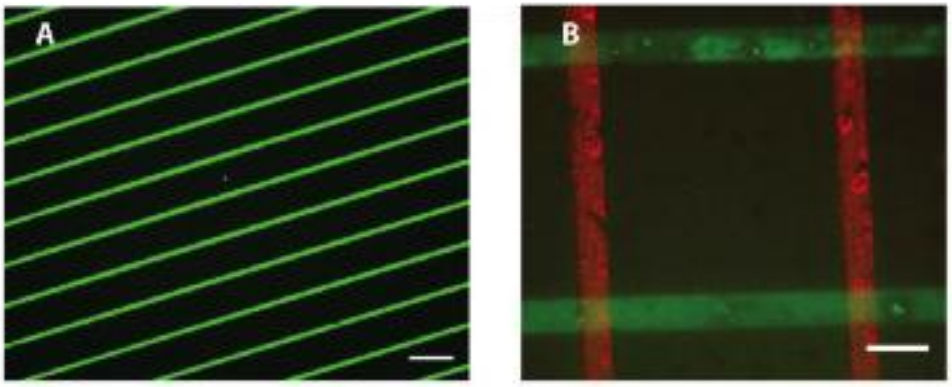

Figure 1.20. Biotinylation of the Streptavidin hydrogel by microcontact printing in A. Dual biotinylation of fibronectin and laminin containing hydrogels in B [34].

2. AIM OF THE STUDY

In this present study a novel orthogonal polyester-based dendron that can 'orthogonally' react with both thiol and azide containing molecules, is synthesized.The dendron consists of an alkene unit at the focal point and alkyne units at the periphery. Hydrogel formation is achieved by Huisgen type Cu catalyzed 'click' reaction of a 2nd generation dendron with PEG diazide as a crosslinker.PEG polymer is used for its unique properties such as high degree of swelling and high biocompatibility. The novel hydrogel network consists of junction points that can be functionalized via 'thiol-ene' click chemistry. (Figure 2.1) The highly porous structure of the network is proved with ESEM and swelling tests.

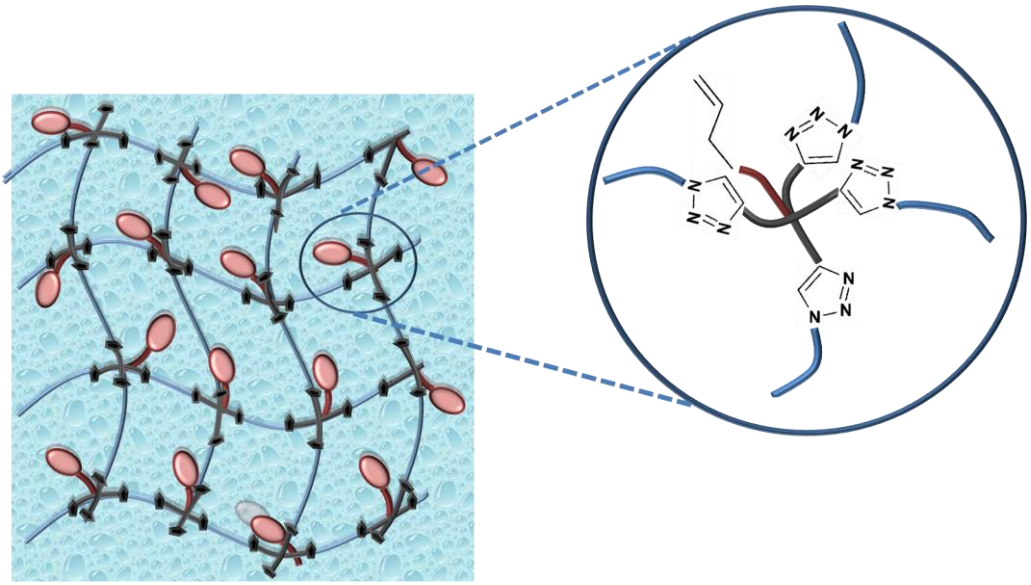

Figure 2.1 Synthesis of photo-functionalizable Hydrogels from orthogonally reactive dendrons.

Candidacy of these novel Hydrogels as reaction platforms is proven by selective post-modification of the alkene functional groups with thiols under UV irradiation. Free alkene units present at the junction points are clicked with thiol containing fluorescent dye and thiol containing ligands for enzyme complexing. Thiol mediated immobilization is demonstrated with photopatterning of the thiol functionalized boron-dipyrromethene (BODIPY) dye on the alkene appended Hydrogels.Efficiency of attachment was screened

via florescence microscope. The ability to choose the binding site of the functional group is proven via photo patterning method. Also enzyme immobilization is shown by covalently attaching the ligand Biotin-SH under UV irradiation and then seeding the fluorescein isothiocyanate (FITC)-Streptavidin, known for its high affinity for Biotin. Binding of the enzyme was also established via fluorescence microscopy. (Figure 2.2)

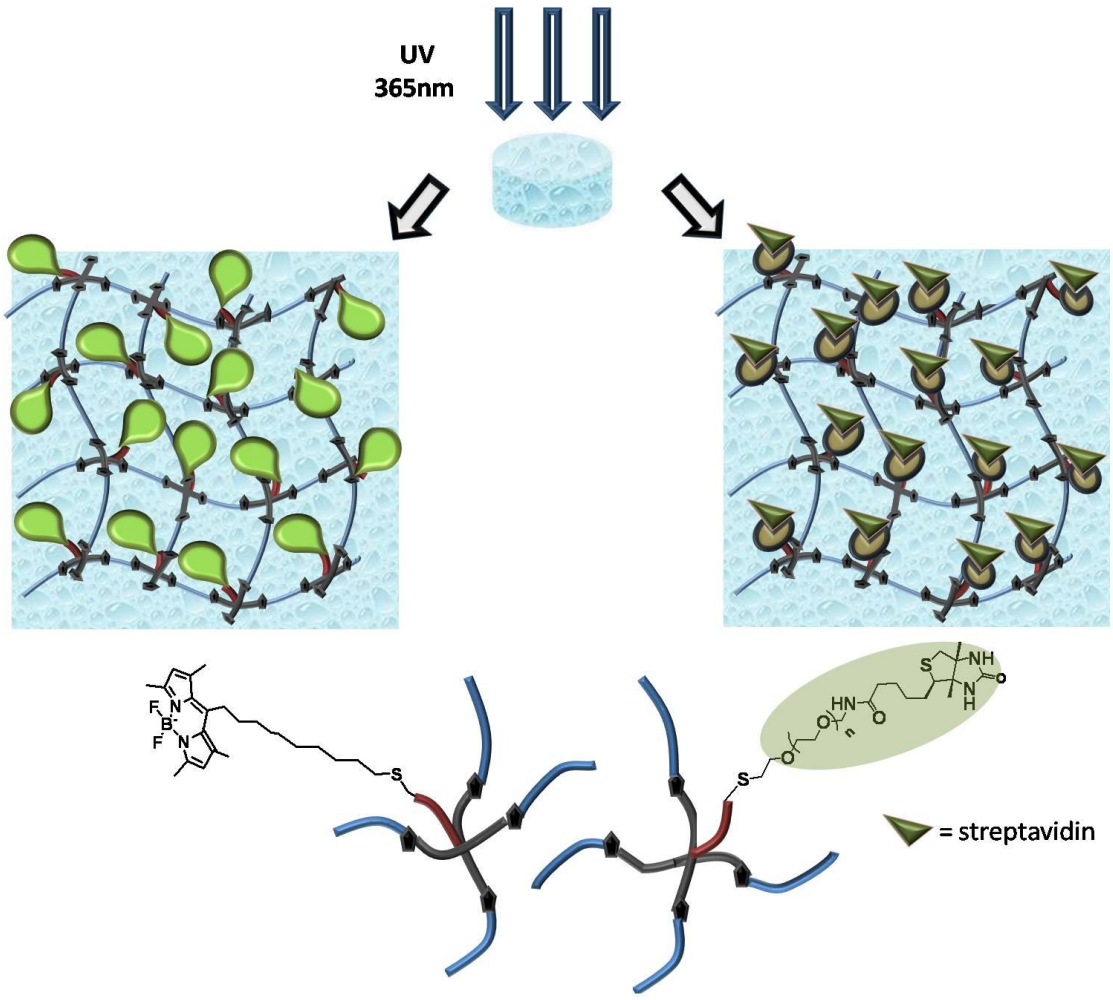

Figure 2.2. Functionalization of the hydrogel via thiol-ene.

Furthermore, these ‘orthogonally’ functionalizable dendron can be used to construct drug delivery systems. A thiol containing targeting group can be attached at the focal point via thiol-ene click reaction, while polymers containing drug molecules can be attached at the arms via [3+2] Huisgen type click reaction.

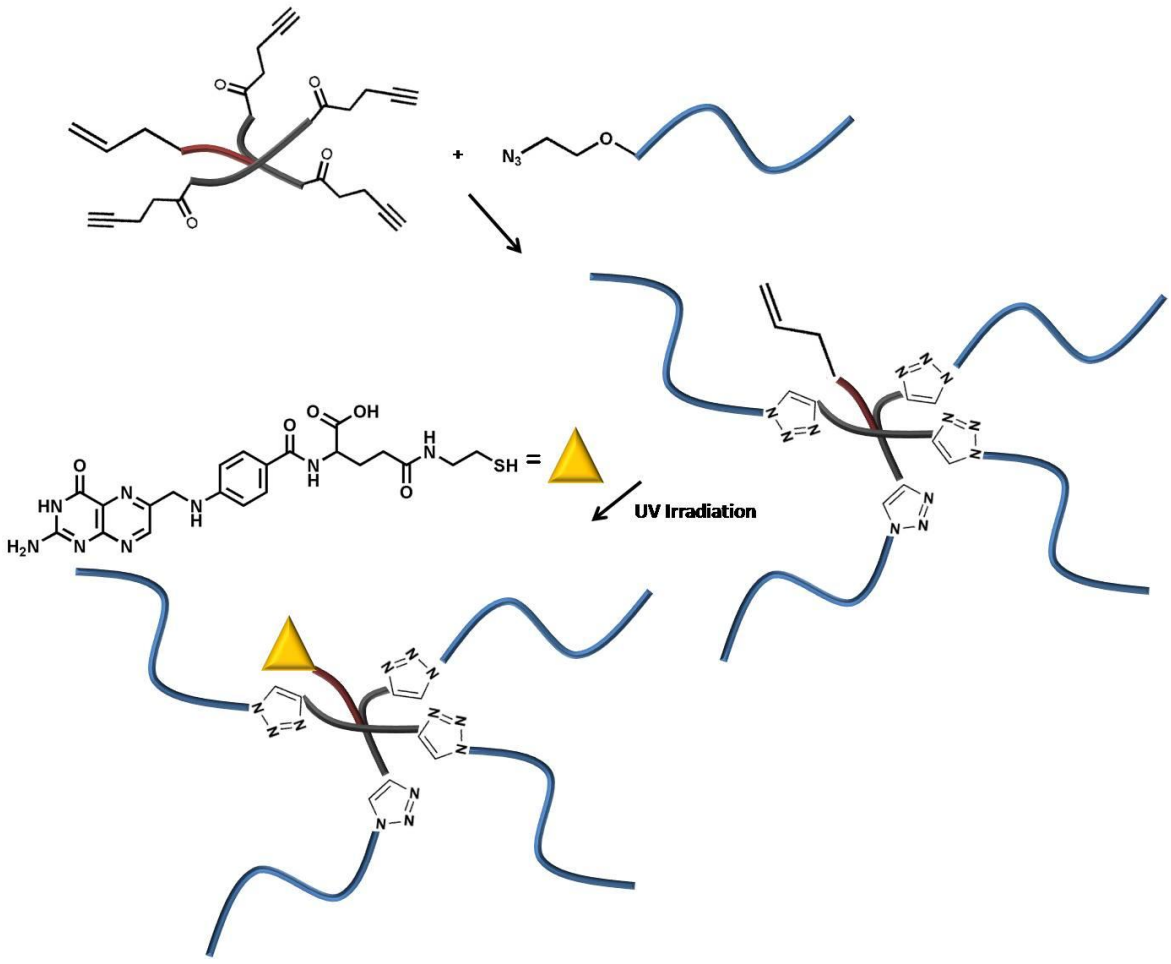

Figure 2.3. Folic Acid attachment on the polymer-dendron conjugates.

3. RESULTS AND DISCUSSION

3.1. Synthesis of the hydrogels

Second generation polyester dendrons with alkene functional group at the focal point(4) is synthesized as shown in Figure 3.1. Stepwise growth of the orthogonal and biodegradable dendrons is achieved via divergent synthesis. Alkyne groups are appended at the dendron periphery for obtaining clickable surface groups. Biocompatible and highly water soluble polyethylene glycol is functionalized with azide groups to yield PEG bis azides (5) with varying molecular weights.Gelation is achieved via click chemistry using PEG-bis azides as the crosslinker between 2nd generation dendrons as shown in Figure 3.2.

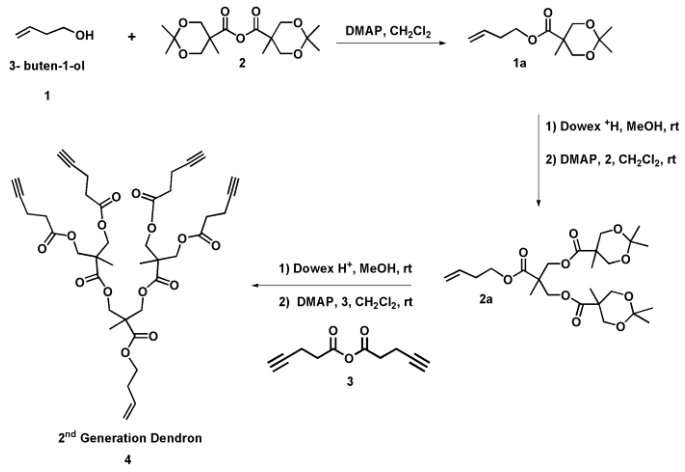

Figure 3.1. Synthesis of 2nd generation dendron (4) as hydrogel precursor.

There are 4 ‘clickable’ alkyne groups which all of them are used in the crosslinking reaction via doubling the equivalence of PEG-bisazide. Active alkyne surface groups of the dendron are available to form triazoles via [3+2] cycloaddition with the azide end groups of the PEG chains which differ in size from 2K to 4K and 6K.

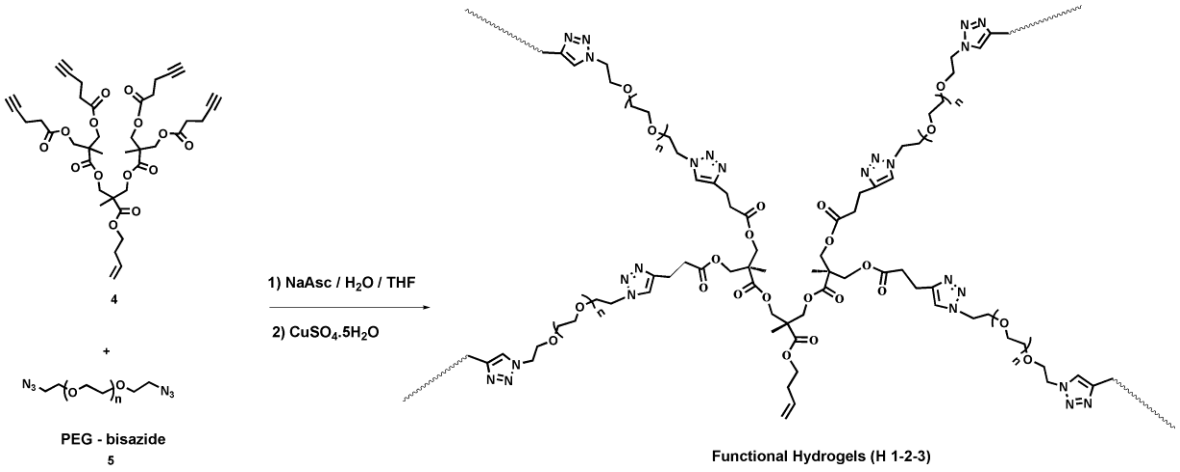

Figure 3.2 Synthesis of Hydrogels (H 1-2-3) via Huisgen type Click Reaction.

The physical and chemical characteristics of the hydrogelsare dependent not only on the efficiency of the click reactionbut also the reaction conditions such as temperature of gelation and type of the solvent. Hydrogels with different PEG units in terms molecular weight are synthesized in both room temperature and 37 ºC. These results are summarized in Table 3.1 and the observations are discussed thereafter.

Table 3.1. Hydrogelation at different temperatures.

Hydrogel	PEG Mn	Gel Conversion at 20 °C	Gel Conversion at 37 °C
H1	2 K	62%	89%
H2	4 K	60%	87%
H3	6 K	53%	84%

According to the Table 3.1, conversion at higher temperatures is much higher due to the increased reactivity. The polymer chain length affects the yield of the reaction. As the PEG chain length decreases the possibility of clicking the azide and alkyne is enhanced.

3.2. Characterization of the hydrogels

Swelling behavior of the hydrogels is probed by recording the water uptake of the hydrogel until it reaches equilibrium. G2-PEG_{2K} (H1) has a more compact structure

leading to decreased ability of swelling. Cross linking density of the hydrogel determined by the cross linker chain length affects the porosity of the network. As the molecular weight of the polymer is increased from 2K to 6K as in hydrogel H3, porosity and swelling capacity is increased (Figure 3.3).

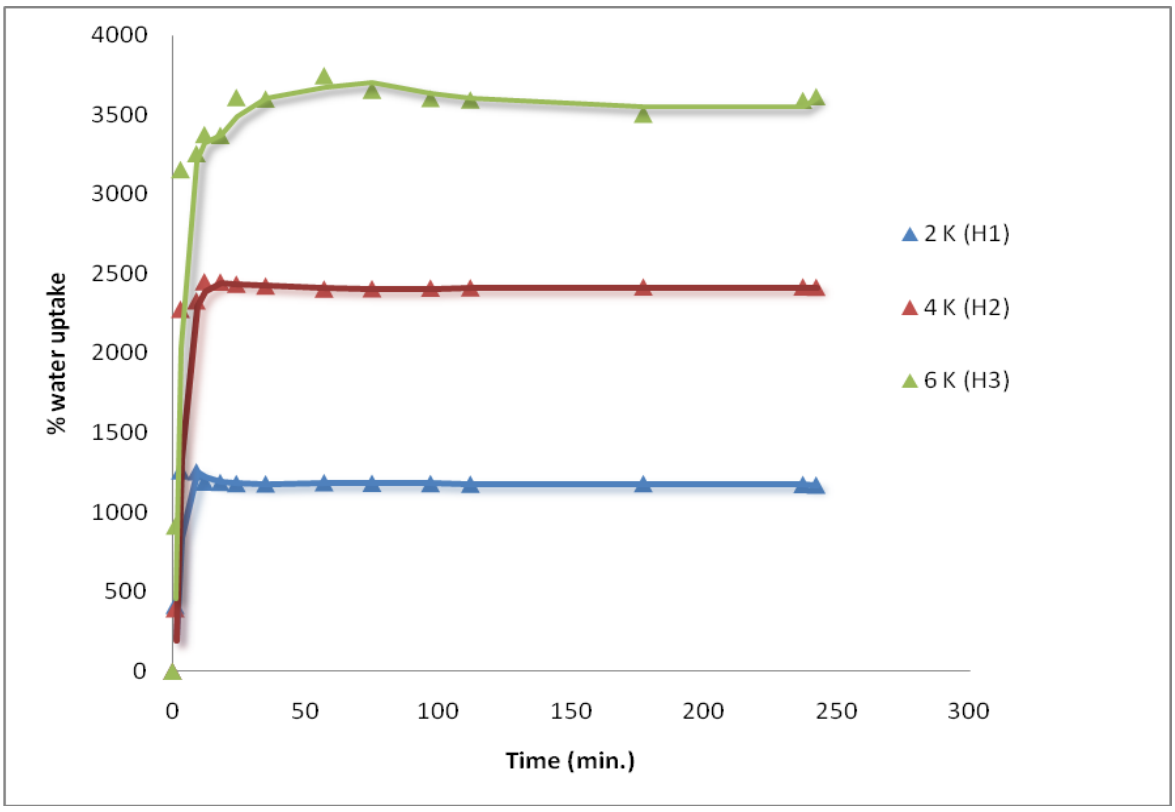

Figure 3.3. Water Uptake Comparison of H1, H2 & H3.

High swelling ability of the hydrogels arises due to highly porous structure of the PEG based dendronized system. The novel 3D geometry due to the dendron linkage points accounts for the increased water uptake capacity (Figure 3.4).

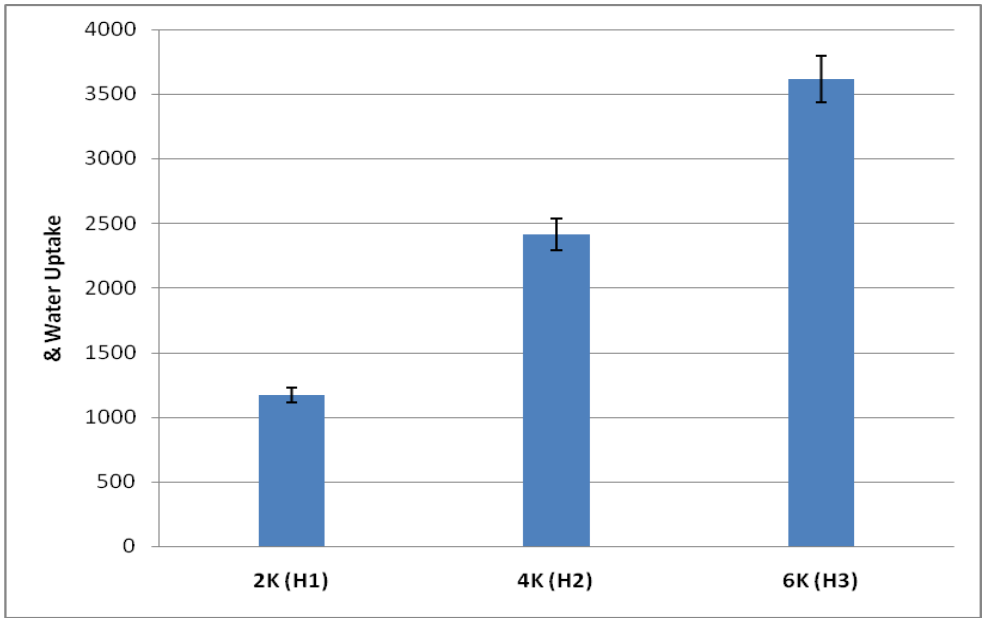

Figure 3.4. Relative water uptake capacities of H1, H2 and H3.

Crosslinking studies are done with hydrogels fabricated with PEG diazides having different chain lengths. PEG units having smaller molecular weights such as 2K are shorter and smaller in size which gives more compact and dense crosslinked structures compared to larger and longer PEG units like 6K forming large pores as shown in the ESEM pictures of the hydrogels in Figure 3.5.

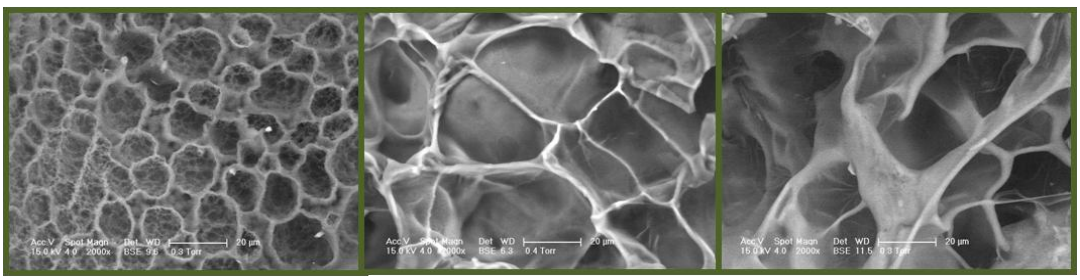

Figure 3.5. Representative ESEM images of H1, H2 and H3 respectively. Image scale bar = 20 micron.

3.3. Functionalization of the hydrogels

Alkene functionality at the core undergoes thiol-ene click reaction with the thiol containing groups upon UV irradiation. Thiol modified fluorescent Bodipy-SH dye is initiated under UV irradiation and selectively attached on the alkene containing surface (Figure 3.6).

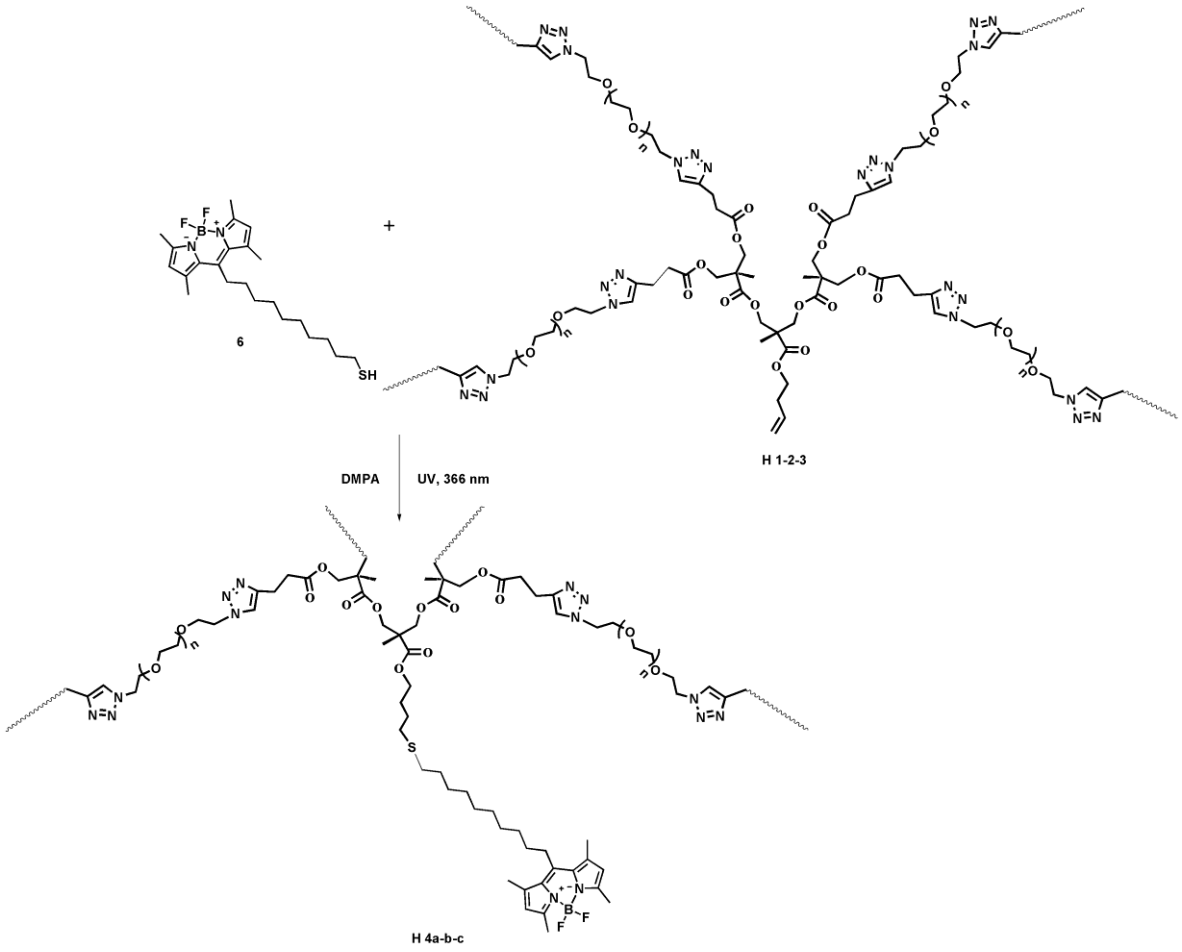

Figure 3.6. Functionalization of H1, H2 and H3 with Bodipy-SH.

As a control a non-functional hydrogel with the butane core is synthesized. Instead of the alkene group at the focal point, butane does not go into thiol-ene click reaction. (Figure 3.7)

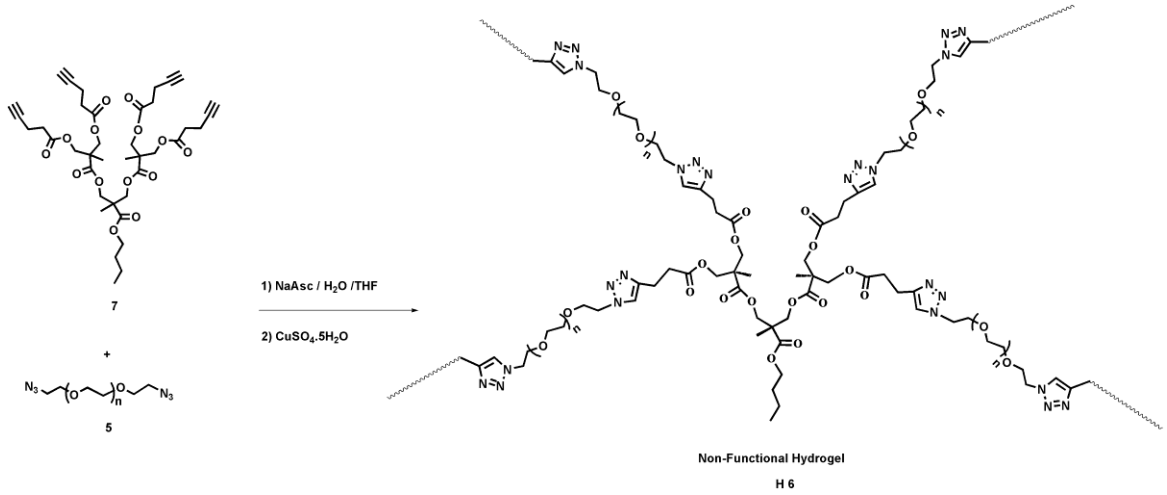

Figure 3.7. Synthesis of non-functional hydrogel (H 6).

The non-functional hydrogel (H6) is treated with the same conditions as H3 and irradiated under UV light for five minutes with the addition of the initiator and after washing of the excess dye molecules, florescent images are taken. Figure 3.8 proves the covalent binding of thiol group to the alkene containing hydrogel whereas butane core does not bind to the any of the thiol group. Excess washing of the dye solution prevents the physisorption of dye molecules.

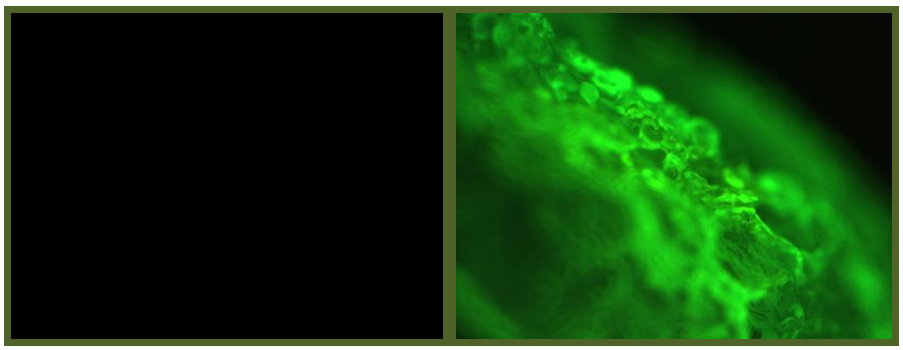

Figure 3.8. Representative images for functionalization of H6 and H3, respectively.

Selectivity of the reaction area on the gel is achieved via photolithography technique. (Figure 3.9)With the photopatterning method on the hydrogels the aim is to show that the reaction works in a controllable manner. Gels are loaded with the dye solution for

homogeneous distribution of the thiol containing dye groups. Photoinitiator is added along with the solution and gels are placed under photomasks.

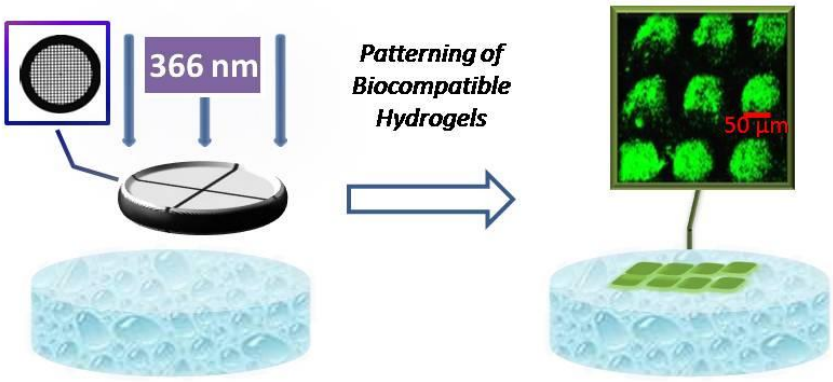

Figure 3.9. Photo patterning of the Hydrogels.

During irradiation process gel shrinkage is observed due to the evaporation of the solvent, THF. After washing-off the unbounded dye molecules with excess solvent, microscopy images are taken via shrinking back the gel to the size on irradiation. Chrome coated photomasks are used with square shapes with side length changing from 0.5 cm to 1 cm. Utilization of TEM grid with squares of 50 µm allows one to obtain square patterns of commensurate length scale (Figure 3.10).

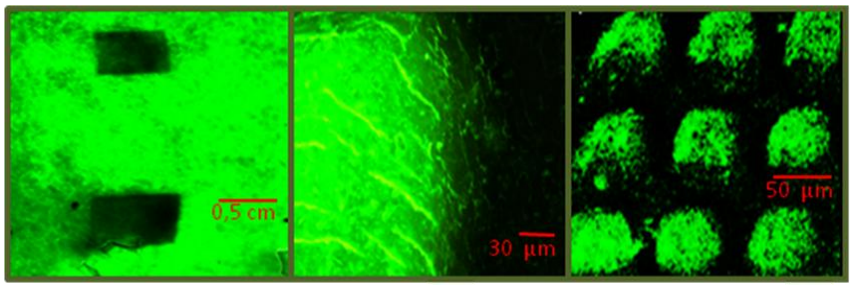

Figure 3.10. Florescence microscopy images of photo patterned hydrogel B3. a) Chrome coated squares having 0.5 cm sides. b) The edge of a patterned and non-patterned area. c) Photolithography with TEM grid.

The neat shapes of the masks are reproduced with negligible errors due to the 3 dimensional geometry and highly absorbent nature of the hydrogel. The representative pictures in Figure 3.10 are taken from the surface of the gels. In Figure 3.10.a masking the squared areas and revealing the rest gives dye attachment on the whole surface except the masked-square areas. In Figure 3.10.b the edge of a square is pictured to show the relatively narrow spatial control on functionalization. Reaction in micron size is shown in Figure 3.10.c with the TEM masking of the hydrogel.

As an alternative, surface functionalization of these hydrogels can be done via micro contact printing under UV irradiation (Figure 3.11). Micro contact printing assay shows the functionalization of the hydrogel can be achieved with fluorescent dye in the shape of the 30 μm width lines using a PDMS stamp having 30 μm features (Figure 3.11). Five minutes of UV irradiation is enough for the reaction between the dye molecules on the stamp in contact with the dry hydrogel surface.

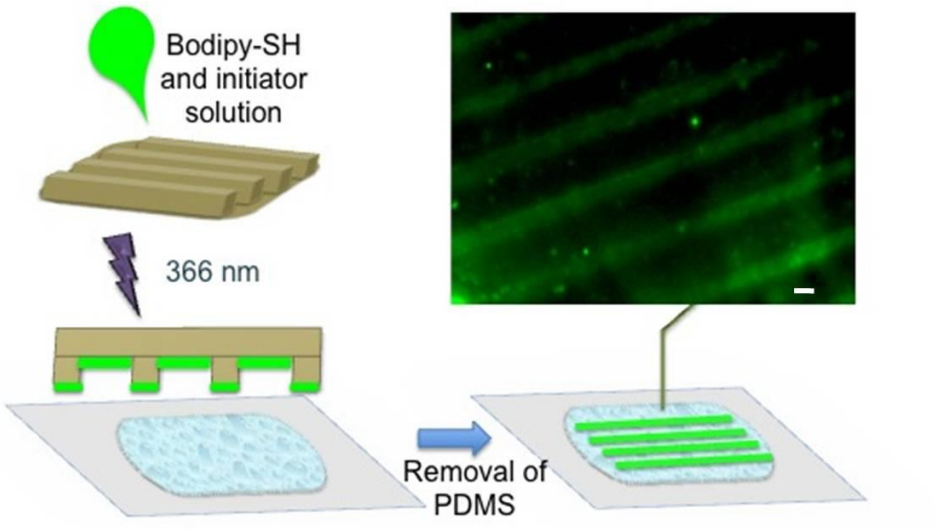

Figure 3.11. Micro contact Printing on the Hydrogels. Representative image of functionalized H3. Image scale bar is 30 micron.

3.4. Characterization of the functionalized hydrogels

The crosslinking density not only affects the water uptake capacity of the hydrogel but also determines the functional group density per area. It is clearly proved that as the functional group density increases the relative intensity increases in quantitative manner. Using a shorter crosslinker increases the available dendron units leading to increment of the available active site per area. Bulk hydrogels of same weights are homogeneously loaded with dye solution and UV irradiation is applied. After the wash-off process florescence intensity is checked from surface of the functionalized gels. 3 different areas from each gel are selected and intensity profiles are taken separately. Control experiment is done without the addition of UV initiator to show that reaction does not occur in the absence of the initiatior uopn UV irradiation. As expected hydrogel with the highest fluorescence intensity is the one having the highest crosslinking density which is constructed with the shortest cross linker, H1. Intensity comparison of the functionalized hydrogels with different cross linkers is shown in Figure 3.12.

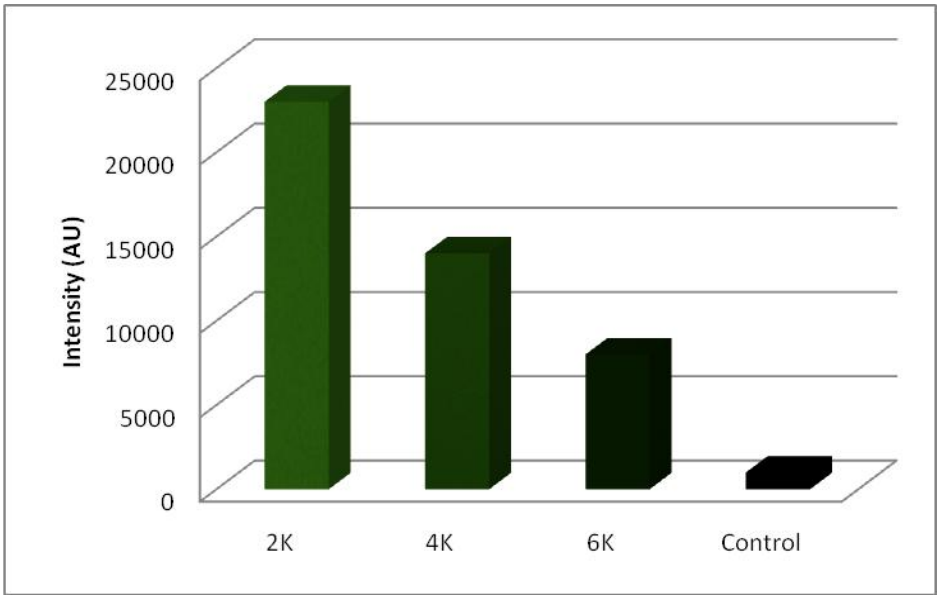

Figure 3.12. Relative fluorescence intensity of H3, H2 and H1 functionalized with Bodipy-SH.

3.5. Bioimmobilization of enzymes on the Hydrogel

Thiol-ene reaction offers metal-catalyst free and mild conditions therefore modification with sensitive biomolecules is applicable. A known ligand biotin thiol is covalently attached onto the bulk hydrogel under UV irradiation in the presence of the initiator. (Figure 3.13)

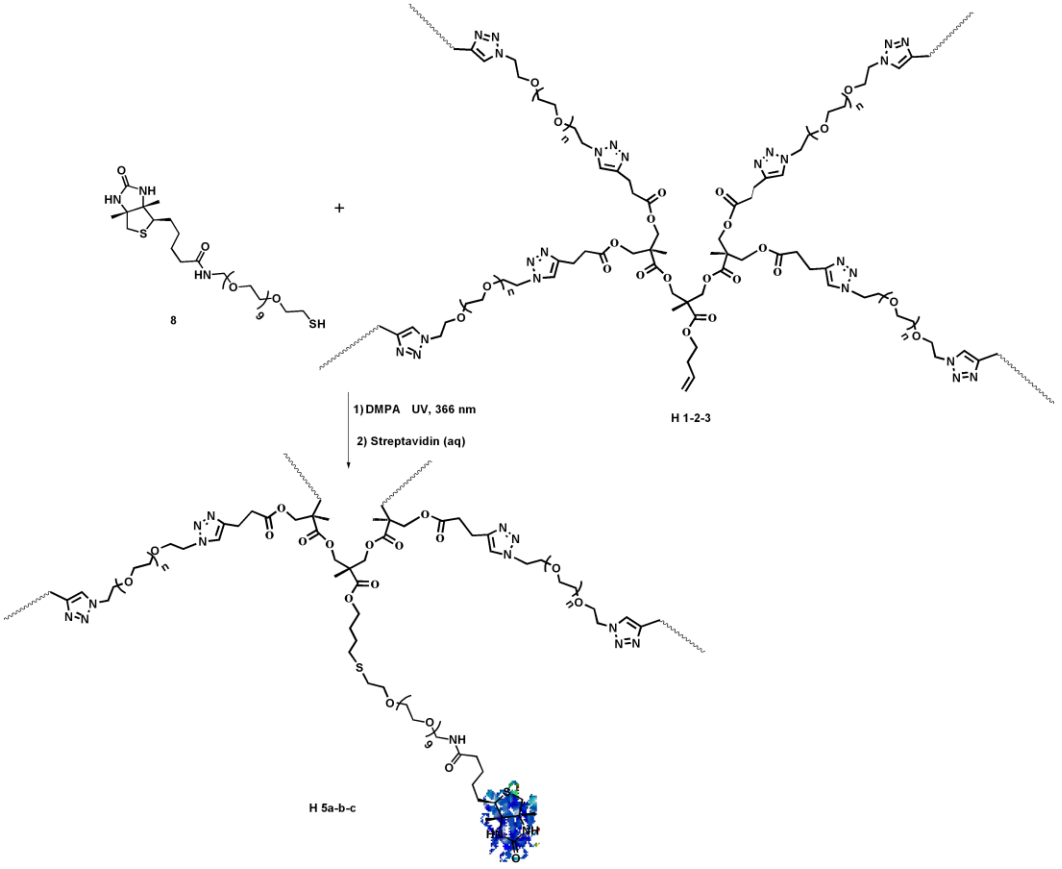

Figure 3.13. Bioimmobilization of Streptavidin on H1, H2 and H3.

Enzyme affinity is shown by using fluorescent FITC-Streptavidin which is known for its high towards biotin. Hydrogels are soaked with the enzyme solution long enough for the complex formation to occur between Biotin and the enzyme. Covalent attachment of biotin is observed with the fluorescence images of enzyme immobilized the hydrogels H1, H2 and H3. These Hydrogels show different fluorescent intensities due to the descending functional group quantity per area (Figure 3.14).

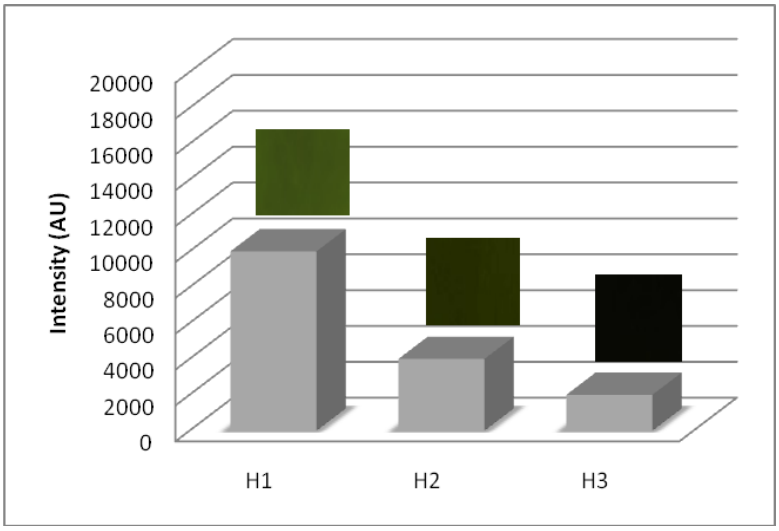

Figure 3.14. Relative fluorescence intensities of Hydrogels H1, H2 and H3 functionalized with FITC-Streptavidin.

3.6. Dendron-Polymer Conjugates as drug carrying agents

To utilize the advantage of orthogonal dendrons as polymeric carriers, alkyne units at the periphery are 'clicked' with PEG-monoazide via [3+2] Huisgen cycloaddition. At the same time, alkene functionality at the focal point is functionalized with thiol containing folic acid under UV irradiation. Folic acid is a known tumor targeting agent due to the over-expression of folate receptors in human tumors. Folic acid conjugated drug carriers has been demonstrated to accumulate into tumor cell by folate receptor-mediated endocytosis [35]. ^{1}H-NMR of the functionalized polymer proves the covalent attachment of folate thiol to the dendron-polymer conjugate. Further investigation is needed and will be done as an ongoing part of this project to get better analytical data.

4. EXPERIMENTAL

4.1. Materials and Methods

All reagents were obtained from commercial sources (Merck, Aldrich and Alfa Aesar) and were used as received unless otherwise stated. Dry solvents (CH_2Cl_2, THF) was obtained from ScimatCo Purification System, other dry solvents were dried over molecular sieves. The dendron and hydrogel characterizations involved ^1H NMR spectroscopy (Varian 400 MHz),FlashEA^R 1112 Series Elemental Analyzer (CHNS Separation Column, PTFE; 2m; 6x5mm), Fourier transform infrared (FTIR) spectroscopy (Perkin Elmer 1600 Series), Scanning Electron Microscopy (ESEM-FEG/EDAX) Philips XL-30 (Philips, Eindhoven, The Netherlands), UV-visible light source (mercury spot lamp; B-100 AP/R) and Zeiss Observer.Z1 inverted fluorescent microscope.

4.2. Synthesis of novel dendrons for Photofunctionalization

4.2.1. Synthesis of 2nd generation dendron (4)

Polyester acid anhydride (2) was prepared according to the literature procedures [36]. DMAP(0.69 g,5.64 mmol) and pyridine(4 mL,51,00 mmol) were added to a flask containing anhydride(2)(7.00 g, 21.20 mmol) and 3-Butene-1-ol (1) (0.85 mL,14.10 mmol) in dry CH_2Cl_2(45 mL) . The reaction stirred at room temperature for 24 hours. Solution was diluted to 100 mL with CH_2Cl_2. Extraction with 3 portions (70 mL) of 1M $NaHSO_4$, then 3 portions (70 mL) of 10% Na_2CO_3solution and (70 mL) with brine respectively gave a colorless solution. Organic part was driedover anhydrous Na_2SO_4 and concentrated in vacuo and high-vacuumed, giving 3 g (93% yield) of ene-G1-acetal (1a)

In order to deprotect the acetal group, ene-G1-acetal (1a) (2.00 g, 8.77 mmol) was dissolved in MeOH (25 mL) and stirred with Dowex X50WX2 (0.05 g) at 40 ºC for 10 hours. Purification was done with column chromatography on silica gel. Eluting with 15:85 ethyl acetate/hexane gradually increasing to 100% ethyl acetate gave the pure compound, 1.2g. (72% yield) (Ene-G1-OH)

Ene-G1-OH (0.60 g,3.20 mmol) ,DMAP(0.69 g,5.64 mmol) and pyridine (4 mL,51.00 mmol) were added to a solution of polyester acid anhydride(2)(3.16 g, 9.50

mmol) in dry CH_2Cl_2(30 mL) . Reaction mixture was stirred for 24 hours then diluted to 70 mL by adding CH_2Cl_2. Extraction with 3 portions (50 mL) of 1M $NaHSO_4$, then 3 portions (50 mL) of 10% Na_2CO_3 solution and (50 mL) with brine respectively gave a colorless solution. Organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo and high-vacuumed, giving ene-G2-acetal (2a) (2.00 g, 63%yield). For deprotection of the acetal groups, ene-G2 acetal (2a) (1.00 g, 2.00 mmol) was dissolved in MeOH (18 mL) and stirred with Dowex (0.02 g) at 40 ºC for 16 hours. Purification was done with column chromatography on silica gel. Eluting with 15:85 ethyl acetate/hexane gradually increasing to 100% ethyl acetate gave a pure compound as 0.75g. (90% yield)(Ene-G2-OH)

For peripheral functionalization of Ene-G2-OH (0.14g,0.3 mmol), DMAP(0.032g,0.26 mmol) and pyridine(0.5 mL,6.30 mmol) were added to a solution of pentynoic anhydride (3) (0.36g,2.00 mmol) in dry CH_2Cl_2 (10 mL)under N_2 and reaction was stirred for 24 hours. Reaction mixture was diluted to 20 mL with CH_2Cl_2 and extracted with 3 portions (14 mL) of 1M $NaHSO_4$, then 3 portions (14 mL) of 10% Na_2CO_3 solution and (14 mL) with brine respectively. Organic layer was dried over anhydrous Na_2SO_4. Crude was purified with column chromatography starting with 10:90 ethylacetate/hexane gradually increasing to 15:85. Pure product was obtained as 0.20 g. (80% yield) (4) ^1H NMR ($CDCl_3$,δ, ppm) 5.81-5.72 (m, 1H), 5.10 (d, 1H, *J* = 16 Hz) 5.08 (d, 1H, *J* = 10 Hz), 4.26-4.19 (m, 12H), 4.16 (t, 2H , *J* = 6.6 Hz), 2.53 (t, 8H, *J* = 6.6 Hz), 2.47 (t, 8H, *J* = 6.6 Hz), 2.38 (dt, 2H, *J* = 6.6 Hz, 6.5 Hz), 1.96 (s, 4H) 1.55 (s, 3H) 1.23 (s, 6H) FTIR (cm^{-1}): 3284, 1726.

4.3. Synthesis of PEG-bisazide (5)

p-TsCl (3.21 g, 16.8 mmol) was added to a flask containing PEG 6K diol (10.00 g, 1.7 mmol), DMAP (2.05 g, 17.0 mmol) and triethylamine (4.68 mL, 33.6 mmol) in CH_2Cl_2 (30 mL). The reaction was stirred at room temperature for 24 h. The mixture was poured onto cold aq. HCl (6M, 150 mL) and was extracted with CH_2Cl_2 (3 x 75 mL). Combined organic layers were dried over Na_2SO_4 and the solvent was removed under *vacuo.* Polymer was dissolved in 5 mL of CH_2Cl_2 and then precipitated in cold diethyl ether(100 mL) and filtered. Dried polymer (5.62 g, 0.8 mmol) was dissolved in DMF (70 mL) and sodium

azide (0.59 g, 8.0 mmol) was added to the solution. The reaction mixture was stirred for 24 h at 60 ^{o}C. The mixture was poured into cold aq HCl (6M, 150 mL) and was extracted with CH_2Cl_2 (3 x 75 mL). Solvent was evaporated under *vacuo*. Polymer was dissolved in 3 mL $CH_2Cl_2$and thenprecipitated in cold diethyl ether.(75 mL) Product dried under high vacuum yielding 6.23 g (90%) of yellowish white solid. FTIR (cm^{-1}): 2869, 2100

PEG(2K)bisazide and PEG(4K)bisazide are synthesized with the same procedure.

4.4. Photofunctionalizible Hydrogels

4.4.1. Synthesis of hydrogels (H 1-2-3) via 'click' reaction

Poly(ethylene glycol)diazide-6K (5) (0.080 g, 0.012 mmol) is added to a small vial containing 2nd Generation Dendron (4) (0.005g, 0.006 mmol) and dissolved in 150 µL THF. 20 µL of aqueous sodium ascorbate (NaAsc) solution (2 M) is added. After addition of 50 µL $CuSO_4.5H_2O$ solution (0.300 M) upon quick sonication gelation occurs in seconds at 37 ºC. After 75 min. of complete gelation, gel is purified with 20 mL of EDTA solution (0.100 M) and H_2O respectively. G2-PEG_{6K} gel conversion based on gel content after lyophilization is 84% for hydrogel H3. FTIR (cm^{-1}): 2877, 1738

Likewise, H1 (87 % yield) and H2 (89% yield) are synthesized according to the same procedure with $PEG_{4K}$and PEG_{2K}respectively.Gelation at room temperature with the same ratios is repeated.

4.4.2. Scanning electron microscopy (SEM) analysis of the hydrogels

The hydrogel samples were equilibrated in H_2O at room temperature and frozen. Frozenswollen hydrogels were lyophilized and scanning electron microscopy was used to characterize the morphology of the hydrogels. The hydrogel was immersed in liquid nitrogen and broken and images were taken using ESEM-FEG/EDAX Philips XL-30 (Philips, Eindhoven, The Netherlands) instrument using an accelerating voltage of 10 kV.

4.4.3. Swelling studies of the hydrogels

The swelling behavior of the hydrogels was characterized as a function of time. A circular piece of purified and dried hydrogel was transferred to a flask containing 30 mL of distilled water at room temperature. The mass of the hydrogel sample was recorded regularly after removing the hydrogel from solution and drying the surface with a filter paper. Measurements were done by calculating the weight gain as a function of immersion time. These experiments were done for a minimum of three samples of a particular hydrogel and repeated at least 3 times with different samples. The ability of swelling was expressed as the swelling ratio percent, W, in which M_w and M_d are wet and dry weights of the samples.

$$W = (M_w - M_d) / M_d \times 100$$

4.5. Photofunctionalization of Hydrogels

4.5.1. Synthesis of fluorescent BODIPY-SH dye (6)

Bromine-end BODIPY precursor(0.215 g, 0.41 mmol) and potassium thioacetate (0,052 mg, 0.45 mmol) were stirred in 25 mL of acetone for 2 h at reflux. After removal of the solvent under vacuo, the orange solid was dissolved in dichloromethane, washed with water several times, and dried over sodium sulfate. The resulting thioacetate was obtained as a red solid and purified with column chromatography eluting with 20% CH_2Cl_2-EtOAc. Then the thioacetate (0.480 mg, 1.07 mmol) was dissolved in 30 mL ethanoland the solution was degassed for 30 minutes with nitrogen gas. After 30 minutes,potassium carbonate was added to the flask and the mixture was gently warmed to ~30°C) After stirringunder N_2 for 4 hours, the contents of the flask was poured into 20 mL of an aqueoussolution of saturated ammonium chloride and extracted with 30 mL dichloromethane.The dichloromethane layer was extracted with water 3 times, dried over sodium sulfate and concentrated. The thiol wasisolated in 85 % yield via column chromatography using 1:1 dichloromethane: hexanes asthe eluant [37].

4.5.2. Functionalization of bulk hydrogel with fluorescent dye (H 4a-b-c)

G2-PEG_{6K} (H3) (0.014g, 2.70 μmole) was treated with 0.3 mL of degassed florescent dye solution (BODIPY-SH in THF,4.50 μmole,0.2M) and 2,2-dimethoxy-2-phenylacetophenone (DMPA) (0.031 mg, 0.12μmole) and placed under UV lamp for 15 minute irradiation.After irradiation, gels (H 4-c) were washed in THF overnight to get rid of the excess dye solution. Fluorescence microscopy images were taken. Same functionalization procedure was applied on H2 and H1 gels with corresponding thiol ratios yielding H 4-b and H 4-a respectively.

4.5.3. Photo assited bioimmobilization of enzymes on the hydrogel surface

G2-PEG_{6K} (H3) (0.014g,2.70 μmol) was treated with 0.5 mL of ligand solution (Biotin-SH in MeOH, 4.1μM)(8) and 2,2-dimethoxy-2-phenylacetophenone (DMPA) (0.200 mg, 0.78 μmole) and placed under UV for 15 minute irradiation.After irradiation, gels were washed in MeOH overnight to get rid of the excess precursor solution. Enzyme bioimmobilization was applied on the functionalized hydrogels with FITC-labeled Streptavidin solution (0.1 mg/ml of PBS buffer, pH 7.4) in the dark for 15 minutes. Fluorescence microscopy images were taken after excess washing with water.(H 5a)

4.5.4. Photolithography on the surface of the hydrogel

G2-PEG_{6K} (H3)(0.014g,2.70 μmole) was treated with 0.3 mL of florescent dye solution(BODIPY-SH in THF, 4.5 mmole, 0.2 M) and 2,2-dimethoxy-2-phenylacetophenone (DMPA) (0.031g,0.12μmole) and placedbetween the chrome coated-patterned mask and cover glass for 15 minute UV irradiation.After photopatterning, gels were washed in MeOH overnight to get rid of the excess dye solution. For TEM functionalization hydrogels were ‘clicked’ in a larger vial to get thinner surfaces. TEM grid was placed tightly on top of the gel surface along with the dye and initiator solution. Fluorescence microscopy images were taken.

4.5.5. Micro contact printing on the hydrogel surface

Spin coating technique was applied to achieve the desired thickness of the gel on the glass surface. PEG (6K)-bisazide (0.04 g, 0.006 mmol) was added to a small vial containing 4 (2.50 mg, 0.003 mmol) in 90 μL THF. To the clear solution 3 μL of aqueous sodium ascorbate (NaAsc) solution (2 M) was added. After addition of 30 μL CuSO4.5H2O solution (0,3 M) the mixture was transferred onto a glass surface and spin coated first with 600 rpm for 15 seconds then 500 rpm for 30 seconds. After 75 min. of complete gelation surface was washed gently with 20 mL of EDTA solution (0.1M) and H_2O respectively. Microcontact printing was used to functionalize the gel surface. 0.5 mL of fluorescent dye solution (BODIPY-SHin THF, 1.10 μmol, 0.1M) and 2,2-dimethoxy-2-phenylacetophenone (DMPA) (0.01 mg, 0.06 μmole) was placed drop wise on to the PDMS plate (1 cm^2, lines of 30 μm thickness separated by 60μm gaps) homogeneously and dried under N_2 stream. PDMS was placed onto the gel surface and irradiated under 365nm UV light for 5 min. Excess dye solution was washed off with THF. Fluorescent microscopy images were taken.

4.5.6. Synthesis and functionalization of non-functional hydrogel(7)

Polyester acid anhydride (2) was prepared according to the literature procedures.[32] DMAP (0.69 g,5.64 mmol) and pyridine(4 mL,51 mmol) were added to a flask containing anhydride(2)(7.00 g, 21.20 mmol) and butanol (0.85 mL,14.1 mmol) in dry CH_2Cl_2(45 mL) . The reaction stirred at room temperature for 24 hours. Solution was diluted to 100 mL with CH_2Cl_2. Extraction with 3 portions (70 mL) of 1M $NaHSO_4$, then 3 portions (70 mL) of 10% Na_2CO_3 and (70 mL) with brine respectively gave a colorless solution. It was dried over Na_2SO_4 and concentrated in vacuo and high-vacuumed, giving 3g(93% yield)of butane-G1-acetal. Deprotection and further divergent growth of the first generation dendron was done in a same manner as in 2^{nd} Generation Dendron (7). Hydrogel synthesis procedure similar to photofunctionalizable hydrogels was followed. Functionalization of these Hydrogels with thiol containing BODIPY-SH dye was carried out in a manner similar to the alkene appended hydrogels (H1 –H3). After subjecting the Hydrogels to washing

protocols to remove unbound dyes, samples were analyzed with fluorescence microscope.

4.6. Photo functionalizable Dendron-Polymer Conjugates

4.6.1. Synthesis of dendron-polymer conjugates

PEG_{750}-monoazide (10) was prepared according to the literature procedure. [38] Compound 4 (0.08 g, 0.10 mmole) was dissolved in X mL dry THF. PEG_{750}-monoazide (0.37 g, 0.48 mmole), Cu(I)Br (6.20 mg, 0.04 mmole) and N,N,N',N',N''-pentamethyldiethylenetriamine (PMDETA) (9 μL, 0.04 μmole) were dissolved in 5 mL dry THF in a separate flask. Both solutions were mixed under N_2 and the mixture was stirred at 50 °C for 24 hours. The product was filtered through Al_2O_3 and then precipitated in ether to give 9 (0.25 g, 56 %) as a light yellow viscous liquid. GPC revealed a single monomodal peak M_n = 4044, PDI = 1.07

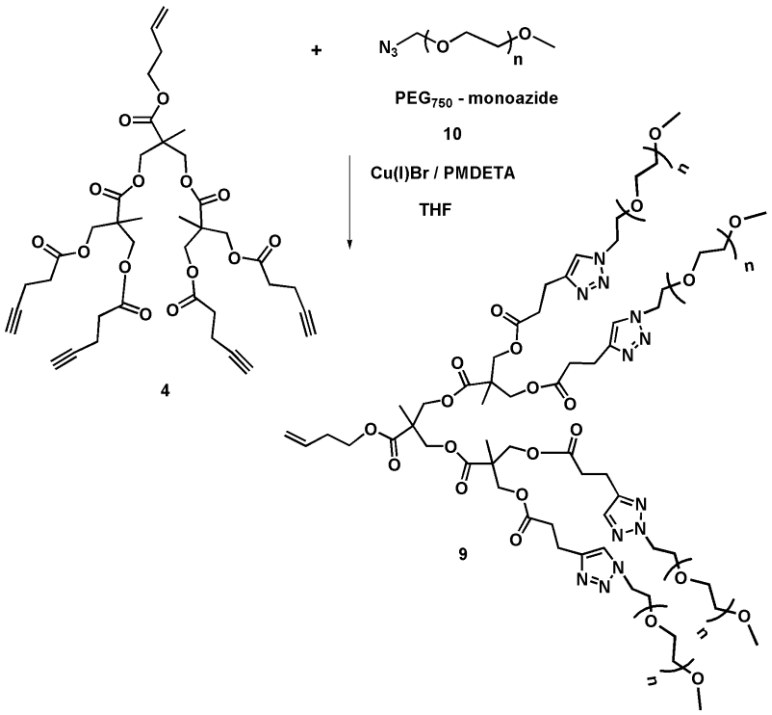

Figure 4.1. Synthesis of dendron-polymer conjugates (9).

4.6.2. Thiol conjugation of Folic Acid

Folate thiol was synthesized according to the literature procedure [8]. Folic Acid (11) (0.5 g, 1.6 mmol) was added into a mixture of anhydrous DMSO (10 mL) and TEA (0.25 mL) and allowed to dissolve in the stirring mixture under anhydrous conditions in the dark overnight. Then, the solution was mixed with DCC (0.23 g, 1.6 mmol) and NHS (0.13 g, 1.6 mmol) and stirred in the dark for 18 h. The side product,dicyclohexylurea, precipitated and was removed by filtration. Mixture is precipitated into 3:7 volume ratio mixture of cold acetone and ether (70 mL). Precipitate is filtered and vacuum-dried. Pure Folate- NHS (12)is obtained. (0.5 g, 82%) Folate NHS (12) then dissolved into 2:1 volume ratio mixture of DMSO and TEA (0.7 mL). An equal molar amount of 2-aminoethanethiol (0.13 g, 1.6 mmol) was added to the mixtureand the reaction was carried out under an anhydrous condition overnight. The product, FA-SH (13), was separated out by precipitating into 3:7 volume ratio mixture of cold acetone and ether (70 mL). Precipitate is filtered and vacuum-dried. (0.4 g, 87%)

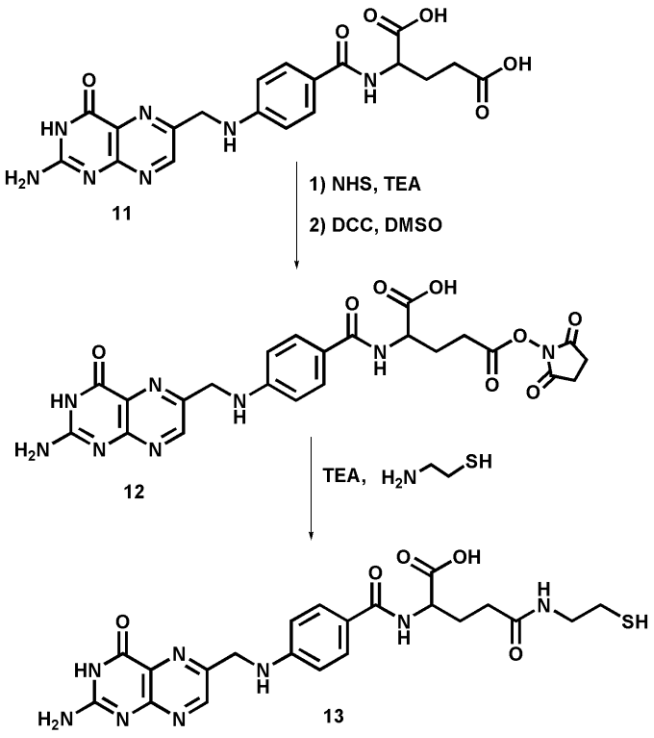

Figure 4.2. Synthesis of Folate Thiol (13).

4.6.3. Photo functionalization of dendron-polymer conjugates

Compound 9 (0.01g, 2.4 μmole) was dissolved in 1 mL DMSO and mixed with folate thiol (13) (8.00 mg, 14.8 μmole) and DMPA (0.024 mg, 0.96 μmole). Reaction is placed under UV lamp and irradiated for 20 min. After removal of the solvent under vacuo, mixture is dissolved in $CHCl_3$ and filtered through sintered glass. Removal of the excess folate thiol gave compound 14 (0.09 mg, 90 %).

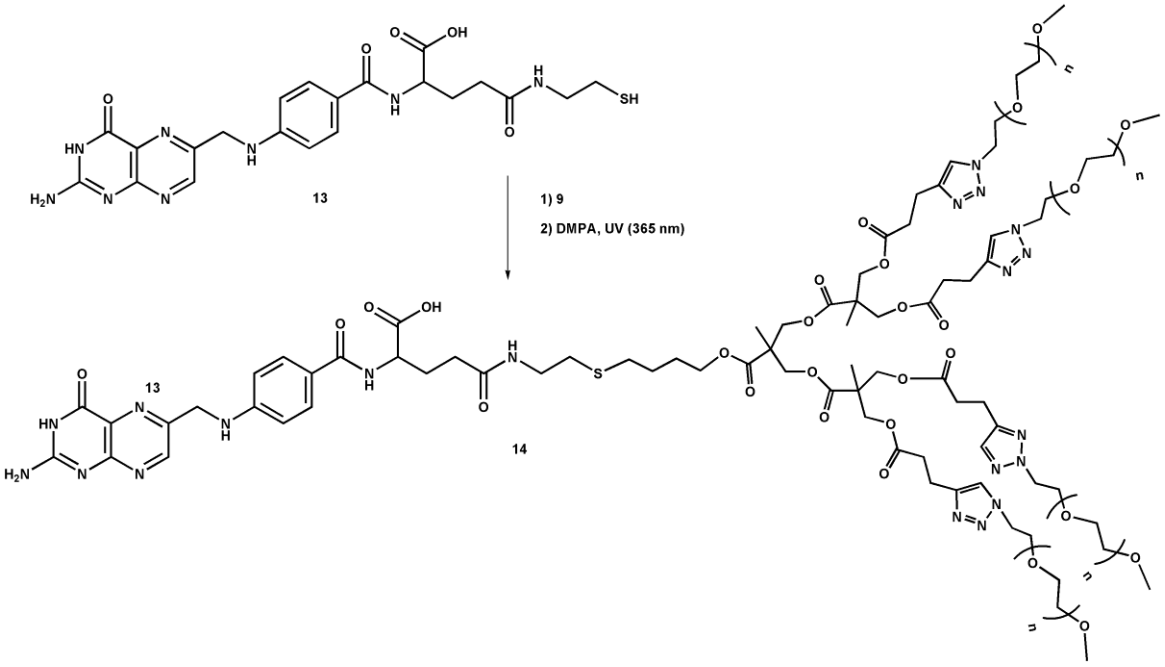

Figure 4.3. Photo Functionalization of dendron-polymer conjugates.

5. CONCLUSIONS

Novel structured hydrogel is fabricated using polyester dendrons as the branching points of bioinert PEG crosslinking agents. Huisgen type Cu catalyzed click reaction is used to build the hydrogel matrix and thiol-ene click reaction is used to further functionalize the hydrogels. Thiol-ene chemistry is shown to be a powerful tool for spatially controllable functionalization of these materials via photolithography technique. Bioimmobilization of Streptavidin enzyme shows that novel dendron based hydrogel is a suitable candidate for bioimmobilization platfrom. Alkene containing dendron-polymer conjugates are proven to be alternative targeting agents via attaching folate thiol, a known tumor cell targeting agent.

APPENDIX

^{1}H NMR spectrasof newly synthesized products FTIR data of hydrogels are included.

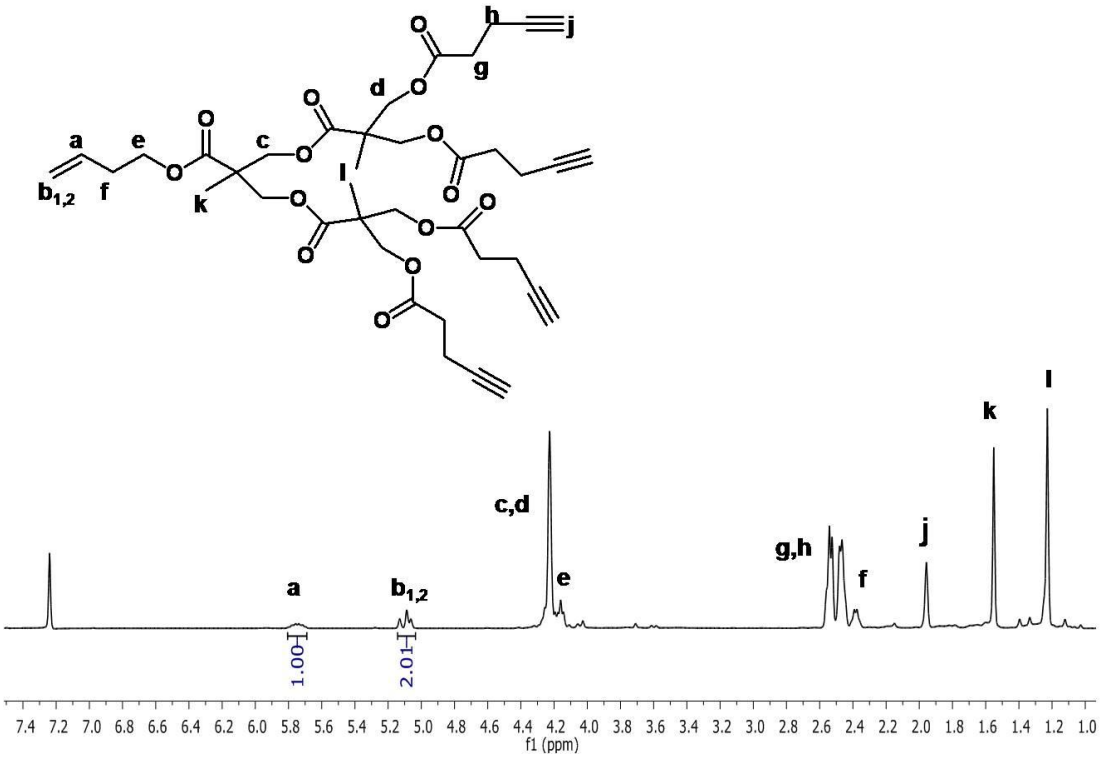

Figure A.1. ^{1}H NMR spectrum of 4.

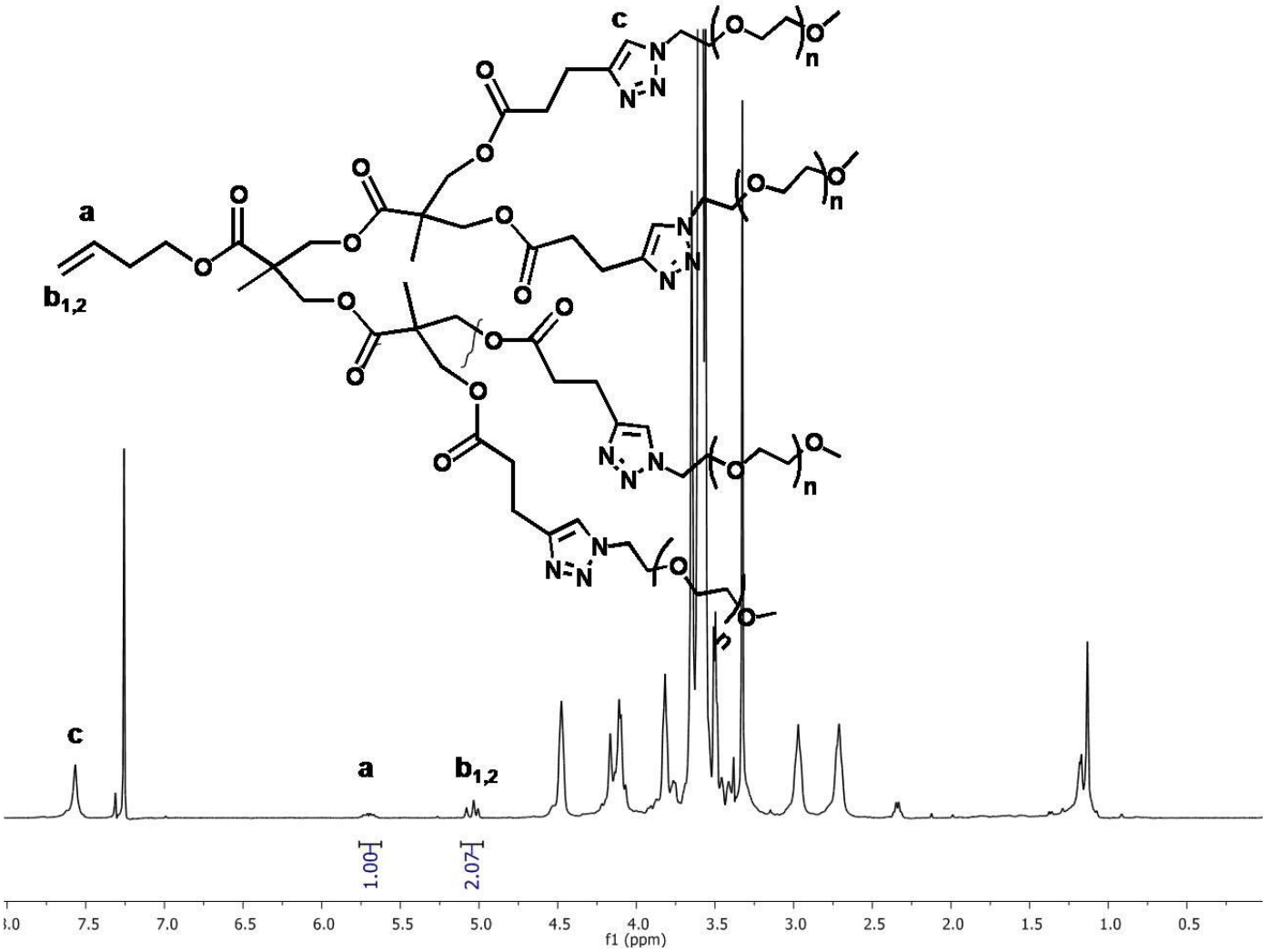

Figure A.2. ^{1}H NMR spectrum of 9.

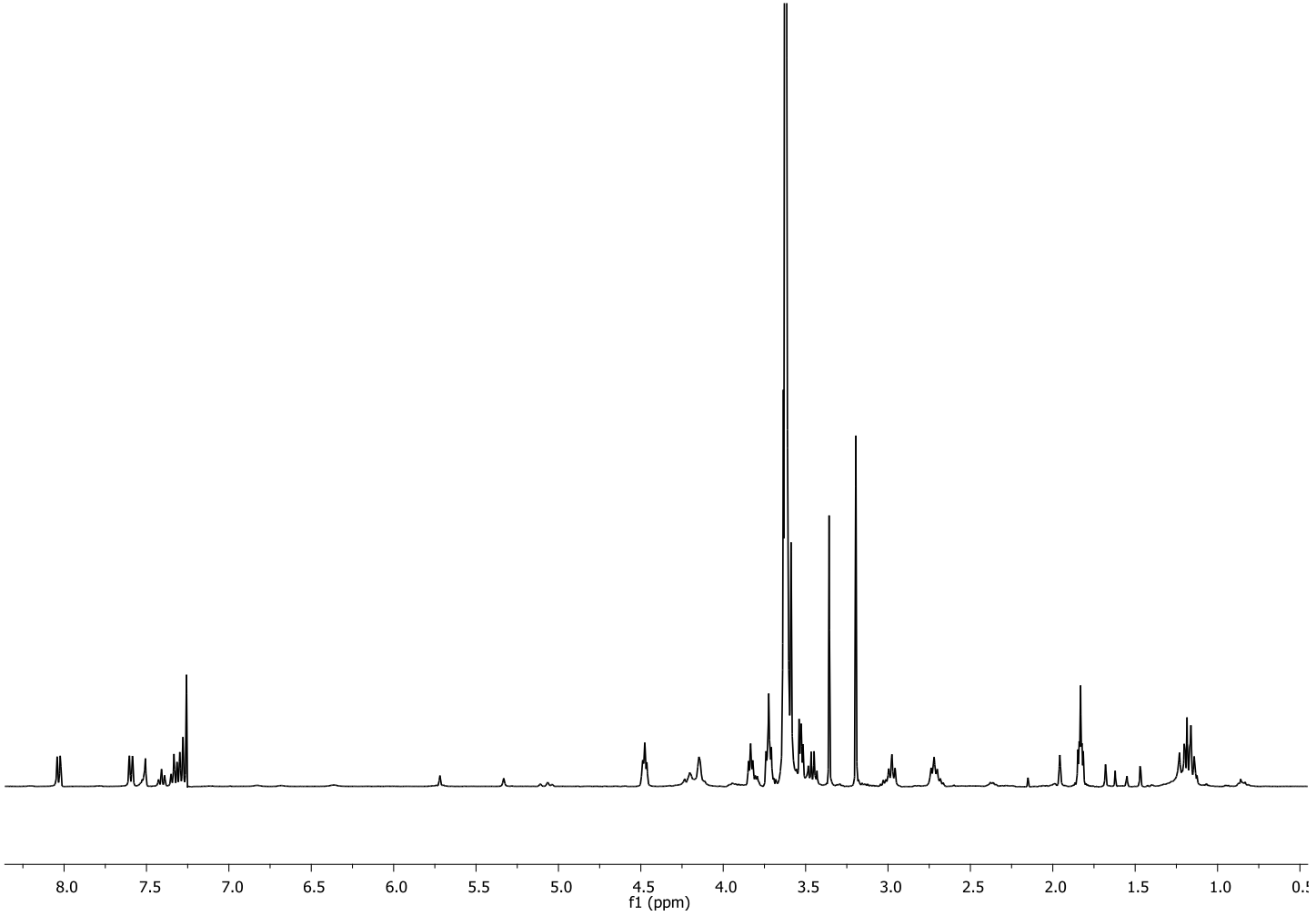

Figure A.3. ^{1}H NMR spectrum of 14.

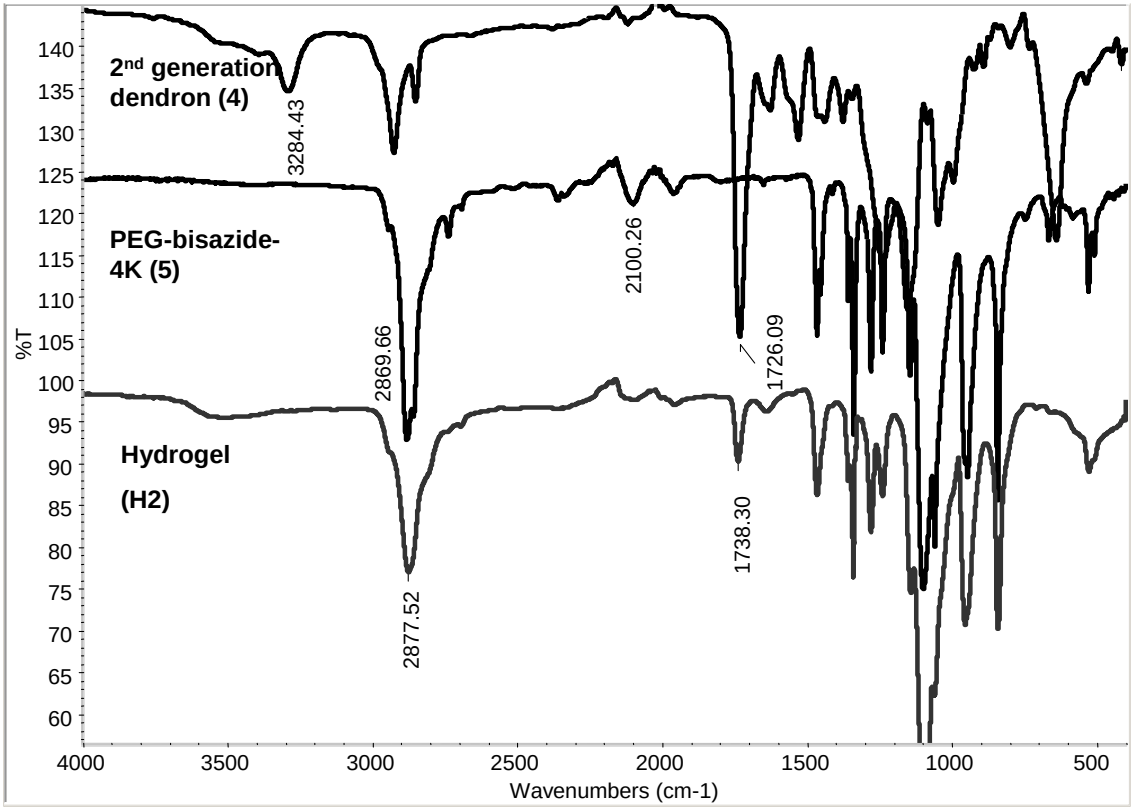

Figure A.4. FTIR Spectra of Hydrogel (H2).

REFERENCES

1. Kosif, I., E. J. Park and R. Sanyal, 'Fabrication of Maleimide Containing Thiol Reactive Hydrogels via Diels-Alder/Retro-Diels-Alder Strategy', *Macromolecules*, v. 43, pp. 4140–4148, 2010

2. Lin, C.C., K. S.Anseth, 'PEG Hydrogels for the Controlled Release of Biomolecules in Regenerative Medicine', *Pharm. Res.*, v. 26, pp. 631-643, 2009

3. Quick, D. J., K. S. Anseth, 'Delivering DNA from Photocrosslinked, Surface Eroding Polyanhydrides', *Journal of Controlled Release,* v. 97, pp. 333– 343, 2004

4. Berger, J., M.Reist, J. M.Mayer, O.Felt, N. A.Peppas and R.Gurny, 'Structure and Interactions in Covalently and Ionically Crosslinked Chitosan Hydrogels for Biomedical Applications', *Eur. J. Pharm. Biopharm.,* v. 57, pp. 19–34, 2004

5. Vashuk, E. V., E. V. Vorobieva, I. I. Basalyga and N. P. Krutko, 'Smart Hydrogel Modeling', *Mat. Res. Innovat.*, v. 4, pp. 350–352, 2001

6. Hoffman, A. S., ' Hydrogels for Biomedical Applications', *Adv. Drug Delivery Rev.*,v. 43, pp. 3–12, 2002

7. Sahiner, N.,'Hydrogel Nanonetworks with Functional Core-Shell Structure', *Eur. Polym. J.,* v. 43, pp. 1709-1717, 2007

8. Thgot n.d., Resorbable Hydrogels, Innovent Biomaterials Department, Polysaccharide derivatives, accessed 30 May 2011, <http://www.thgot.de /en /INNOVENT /Departments/Biomaterials /Polysaccharide-derivatives- /Resorbable-hydrogels-_4661/>

9. Xing,B., C. W. Yu, K. H. Chow,P.L. Ho, D. Fu and B. Xu, 'Hydrophobic Interaction and Hydrogen Bonding Cooperatively confer a Vancomycin Hydrogel', *J. Am. Chem. Soc.*, v. 124, pp. 14846, 2002

10. Lu, Y., P. S. Low, 'Folate Mediated Delivery of Macromolecular Anticancer Therapeutic Agents', *Adv. Drug Delieryv. Rev.*, v. 54, pp. 675-693, 2002

11. Hongchen, D., K. Matyjaszewski, ‘ One-Pot Synthesis of Robust Core/Shell Gold Nanoparticles’, *Macromolecules*, v. 43, pp. 4623-4628, 2010

12. Wei, H. L., Z. Yang, Y. Chen, H. J. Chu, J. Zhu and Z. C. Li, ‘Characterization of N-vinyl-2-pyrrolidone-based Hydrogels Prepared by a Diels-Alder Click Reaction in Water’, *Eur.Polym. J.,* v. 46, pp. 1032, 2010

13. Gupta, N., B. Lin, L. Campos, M. Dimitriou, S. Hikita, N. Treat, M.Tirrell, D. Clegg, E. Kramer and C. Hawker, ‘A versatile Approach to High-throughput Microarrays using Thiol-ene Chemistry’*, Nat. Chem.*, v. 2, pp. 138-145, 2008

14. Martens, P., K. Anseth, ‘Characterization of Hydrogels Formed from Acrylate Modified poly(vinyl alcohol) Macromers’, *Polymer*, v. 41, pp. 7715-7722, 2000

15. Polizzotti B. D, B. D. Fairbanks and K. S. Anseth, ‘Three Dimensional Biochemical Patterning of Click-Based Composite Hydrogels via Thiolene Photopolymerization’, *Biomacromolecules*, v. 9 , pp. 1084, 2008

16. Grinstaff, M. W., ‘Dendritic Macromers for Hydrogel Formation’, *J. Polym. Sci. Part A: Polym. Chem*., v. 46, pp. 383, 2008

17. Kolb, M. W., M.G. Finn and K.B. Sharpless, ‘Click Chemistry’, *Angew. Chem. Int. Ed*., v. 40, pp. 2004-2021, 2004

18. Simsa, E. A., C. A. DeForest and K.S. Anseth, ‘ A Mild, Large Scale Synthesis of 1,3-Cyclooctanedione’, *Tetrahedron Lett*, v. 52, pp. 1871-1873, 2011

19. Joralemon, M.J., R.K. O'Reilly, J.B.Matson, A.K.Nugent, C.J.Hawker and K.L. Wooley, ‘Dendrimers Clicked Together Divergently’, *Macromolecules*, v. 38, pp. 5436-5443, 2005

20. Killops, K. L., L. M.Campos and C. J.Hawker, ‘Development of Thermal and Photochemical Strategies for Thiol-ene Click Polymer Functionalization’, *J. Am. Chem. Soc*., v. 130, pp. 5062–5064, 2008

21. Guhr, K.I, M.D.Greaves and V. M.Rotello, ‘Reversible Covalent Attachment of C-60 to a Polymer Support’, *J. Am. Chem. Soc.,* v. 116, pp. 5997–5998, 1994

22. Wichterle, O., D. Lim, 'Hydrophilic Gels for Biological Use', *Nature*, v. 185, pp. 117, 1961

23. Ossipov, D.A., J. Hilborn,'Poly(Vinyl Alcohol) Based Hydrogels Formed by Click Chemistry', *Macromolecules*, v. 39, pp. 1709-1718, 2004

24. Malkoch, M.; R. Vestberg, N. Gupta, L. Mespouille, P. Dubois, A. F. Mason, J.L. Hedrick, Q. Liao, C. W. Frank, K. Kingsbury and C. J. Hawker, 'Synthesis of well-defined Hydrogel Networks using Click Chemistry', *Chem. Commun*., v. 26, pp. 2774-2776, 2004

25. Dijk, M., F. vanNostrum, W. E. Hennink, D. T. S.Rijkers and R. B. J. Liskamp, 'Synthesis and Characterization of Enzymatically Biodegradable PEG and Peptide-Based Hydrogels Prepared by Click Chemistry', *Biomacromolecules,* v. 11, pp. 1608-1614, 2010

26. Yang, J., M. T. Jacobsen, H. Pan and J. Kopecek, 'Synthesis and Characterization of Enzymatically Degradable PEG-Based Peptide-Containing Hydrogels', *Macromol. Biosci.*, v. 10, pp. 445-454, 2010

27. Degoricija, L.,P. N. Bansal, S. H. M. Sӧntjens, N. S. Joshi, M. Takahashi, B. Snyder and M. W. Grinstaff, 'Hydrogels for Osteochondral Repair Based on Photocrosslinkable Carbamate Dendrimers', *Biomacromolecules,* v. 9, pp. 2863–2872, 2008

28. Altin, H.,I. Kosif and R. Sanyal, 'Fabrication of Clickable Hydrogels via Dendron-Polymer Conjugates', *Macromolecules*, v. 43, pp. 3801-3808, 2010

29. Wosnick, J. H.,M. S. Shoichet, 'Three Dimensional Chemical Patterning of Transparent Hydrogels', *Chem.Mater.,* v. 20, pp. 55, 2008

30. DeFrost, C. A., B. D. Polizzotti and K. S. Anseth, 'Sequential Click Reactions for Synthesizing and Patterning Three-Dimensional Cell Microenvironments', *Nat. Mater.,v.* 8, pp. 659, 2011

31. Laibinis, P. E., J. J. Hickman, M. S. Wrighton and G. M. Whitesides, 'Orthogonal Systems for Self-Assembled Monolayers', *Science*, v. 245, pp. 845-7, 1989

32. Coq, N., T. v. Bommel, R. A. Hikmet, H. R. Stapert and W. U. Dittmer, 'Self-supporting Hydrogel Stamps for the Microcontact Printing of Proteins', *Langmuir, v.*23, pp. 9, 2007

33. Chirra, H. D., D. Biswal and J. Z. Hilt, 'Controlled Synthesis of Responsive Hydrogel Nanostructures via Microcontact Printing and ATRP', *Polym. Adv. Technol.*,v. 6, pp. 773-780, 2011

34. Hynd, M. R.,J. P. Frampton, M. R. Burnham,D. L. Martin,N. M. Dowell-Mesfin,J. N. Turner and W.Shain, 'Functionalized Hydrogel Surfaces for the Patterning of Multiple Biomolecules', *J Biomed Mater Res Part A*, v. 81A, pp. 347–354, 2007

35. Prabaharan, M., J. J. Grailer, D. A. Steeber and S. Gong, 'Thermosensitive Micelles Based on Folate Conjugated Poly(N-vinylcaprolactam)-block-Poly(ethylene glycol) for Tumor Targeted Drug Delivery', *Macromol. Biosci.*,v. 9, pp. 744-753, 2009

36. Malkoch, M., E. Malmström,A. Hult, 'Rapid and Efficient Synthesis of Aliphatic Ester Dendrons and Dendrimers', *Macromolecules*, v. 35, pp. 8307-8314, 2002

37. Shepherd, J. L., A. Kell, E. Chung, C. W. Sinclar, M. S. Workentin and D. Bizzotto, 'Selective Reductive Desorption of a SAM Coated Gold Electrode Revealed Using Florescence Microscopy', *J. Am. Chem. Soc.*, v. *126*, pp. 8329–8335, 2004

38. Kose, M. M., G. Yesilbag and A. Sanyal, 'Segment Block Dendrimers via Diels-Alder Cycloaddition'*, Org. Lett*., v. 10, pp. 2353-2356, 2008