FUNCTIONAL MACROMOLECULAR PLATFORMS: HYDROGEL AND SOLUBLE POLYMER-BASED MATERIALS FOR BIOMEDICAL APPLICATIONS

by

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Dedicated to my dear family

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ABSTRACT

FUNCTIONAL MACROMOLECULAR PLATFORMS: HYDROGEL AND SOLUBLE POLYMER-BASED MATERIALS FOR BIOMEDICAL APPLICATIONS

Design, fabrication and applications of novel hydrogel and soluble polymer-based macromolecular platforms employing functional polymer building blocks are described. Several click chemistry-based transformations and post-polymerization modification techniques have been evaluated in fabrication of chemically cross-linked hydrogels. In conjunction with novel design of reactive polymeric materials, hydrophilic linear polymers with clickable functionalities were synthesized and used in various applications. In the first study, fabrication of well-defined chemically cross-linked poly(ethylene glycol) (PEG)-based hydrogels using the thiol-maleimide addition reaction is reported. Hydrogels obtained by crosslinking of homobifunctional PEGs and a multifunctional β-cyclodextrin-based (β-CD) crosslinker were evaluated in post-gelation modification reactions. In the second project, fabrication of poly(ethylene glycol)-based chemically cross-linked hydrogels containing discrete β-CD units is outlined. Various hydrogels obtained by radical thiol-ene reactions were investigated in terms of their physical gel properties as well as uptake and controlled release of a hydrophobic drug molecule. The third project involves the utilization of reversible addition−fragmentation chain-transfer polymerization-mediated homotelechelic and hetero telechelic polymers toward post-polymerization functionalization with thiol-containing molecules, as well as fabrication of hydrogel networks. In the fourth project, design, synthesis and post-polymerization functionalization of linear PEG-based polytriazole copolymers containing thiol-reactive functional groups as pendant side chains is described. These polymers were utilized in functionalization via click chemistry-based transformations and fabrication of chemically cross-linked functionalizable hydrogels. The fifth project outlines the synthesis of novel thiol-reactive polymers containing a catechol moiety and demonstrates their application towards surface modification of magnetic nanoparticles. The final project describes the synthesis of novel polymers containing activated carbonate groups at side chains and demonstrates their application towards the preparation of polymer-drug conjugates.

ÖZET

FONKSİYONEL MAKROMOLEKÜLER PLATFORMLAR: BİYOMEDİKAL UYGULAMALAR İÇİN HİDROJEL VE ÇÖZÜNÜR POLİMER TABANLI MALZEMELER

Fonksiyonel polimer yapı taşlarının kullanımını içeren yeni tip hidrojel ve çözünür polimer tabanlı makromoleküler platformların dizayn, üretim ve uygulamaları araştırılmıştır. Çeşitli klik kimyası tabanlı dönüşümler ve polimerizasyon sonrası modifikasyon yöntemleri kullanılarak kimyasal çapraz bağlı hidrojellerin üretimi gerçekleştirilmiştir. Ayrıca yeni tasarımlı reaktif polimerler sentezlenmiş ve değişik uygulamalarda kullanılmışlardır. İlk çalışmada, tiyol-maleimid katılma tepkimesini kullanarak, poli(etilen glikol) (PEG)-tabanlı, kimyasal çapraz bağlı, iyi tanımlanmış ağ yapıdaki hidrojellerin üretimi açıklanmıştır. Homobifonksiyonel PEG polimerleri ve multifonksiyonel β-siklodekstrin tabanlı çapraz bağlayıcı ile hazırlanan hidrojeller jel sonrası modifikasyon tepkimelerinde kullanılmıştır. İkinci projede PEG-tabanlı, β-siklodekstrin grupları içeren, kimyasal çapraz bağlı hidrojellerin sentezi anlatılmıştır. Radikal tiyol-en tepkimesiyle hazırlanan bu hidrojellerin fiziksel jel özellikleri ile hidrofobik bir ilacı yükleme ve kontrollu salınım özellikleri araştırılmıştır. Üçüncü proje, RAFT polimerizasyonu ile elde edilmiş homotelekelik ve heterotelekelik polimerlerin tiyol içeren moleküllerle polimerizasyon sonrası işlevselleştirme ve hidrojel üretimi çalışmalarını içermektedir. Dördüncü projede, tiyol-reaktif yan dallar içeren, doğrusal PEG bazlı politriazol polimerlerin dizayn, sentez ve polimerizasyon sonrası işlevselleştirme uygulamaları açıklanmıştır. Bu polimerler, klik kimyası tabanlı dönüşümler ve kimyasal çapraz bağlı hidrojellerin sentezinde kullanılmıştır. Beşinci proje yeni tip tiyol rekatif katekol grubu içeren polimerlerin sentezini ve manyetik nanopartiküllerin yüzey modifikasyonunda kullanımını açıklamaktadır. Son projede yeni tip aktifleştirilmiş yan dal karbonat grupları içeren polimerlerin sentezi ve polimer-ilaç konjugatlarının hazırlanmasında kullanılmaları anlatılmıştır.

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LIST OF ACRONYMS/ABBREVIATIONS

AIBN	2,2′-Azobis(2-methylpropionitrile)
β-CD	β-Cyclodextrin
$CDCl_3$	Deuterated chloroform
DA	Diels-Alder
DMAP	4-Dimethylaminopyridine
DMF	Dimethyl formamide
DMPA	2,2-Dimethoxy-2-phenylacetophenone
d-DMSO	Deuterated dimethyl sulfoxide
EDCI	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EtOAc	Ethyl Acetate
FT-IR	Fourier Transform Infrared
GPC	Gel Permeation Chromotography
MeOH	Methanol
MHz	Mega hertz
NMR	Nuclear Magnetic Resonance
PEG	Poly(ethylene glycol)
PEGMEA	Poly(ethylene glycol) methyl ether acrylate
PEGMEMA	Poly(ethylene glycol) methyl ether methacrylate
RAFT	Reversible-addition Fragmentation Chain Transfer Polymerization
rDA	Retro Diels-Alder
SEC	Size-Exclusion Chromatography
TEA	Triethylamine
TGA	Thermo Gravimetric Analysis
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
UV	Ultraviolet

1. INTRODUCTION

1.1. Cyclodextrin Containing Chemically Cross-linked Hydrogels as Biomedical Aids

1.1.1. Hydrogels

Hydrogels are three-dimensional cross-linked polymeric networks that are constructed using hydrophilic monomers or polymers that can absorb pronounced amount water while retaining the network structure and viscoelastic behavior [1–4] (Figure 1.1). The hydrophilic domains of hydrogel network structure are primarily responsible for the hydration in aqueous environment. The degree of hydration/swelling can be tuned by changing various parameters such as molecular composition of hydrogel, crosslinking degree and external stimuli. Crosslinking points throughout the hydrogel network structure are essential to prevent the dissolution of polymer chains into the aqueous phase that would result the collapse of insoluble network structure.

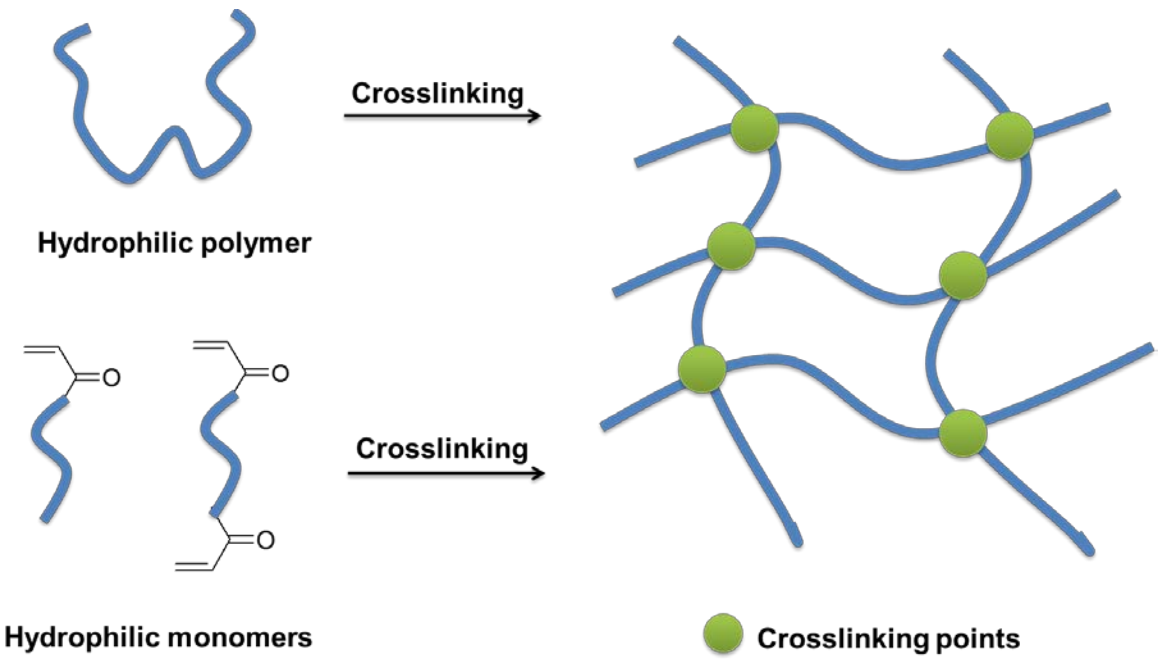


Figure 1.1. Schematic illustration of hydrogel network formation from hydrophilic polymers or hydrophilic monomers.

Depending on the nature of crosslink junctions, hydrogels are divided into two main categories: chemically cross-linked hydrogels and physically cross-linked hydrogels. In chemically cross-linked hydrogels, the network structure is built through permanent junction points that arise upon either crosslinking of polymer chains with each other or bridging the chains with crosslinker molecules. Chemically cross-linked hydrogels are synthesized by either polymerizing of monomers in the presence of a crosslinking agent or crosslinking of polymer chains. The chemical crosslinks can be based on irreversible chemical linkages or reversible chemical linkages that would be broken based on change in conditions (pH, temperature, etc.). In physically cross-linked hydrogels, transient junctions that can be the result of physical factors such as polymer chain entanglements, intermolecular interaction between polymer chains (ionic interactions, hydrogen bonding, and hydrophobic interactions) or physical interactions such as ionic interactions, hydrogen bonds, or hydrophobic interactions results in network formation [5]. Since in chemically cross-linked hydrogels the crosslinking is formed by covalent bonds, these network structures are generally more stable than the networks formed by physically crosslinking. In physical crosslinking, non-covalent interactions between the polymer chains provide the network formation. These interactions are usually weak interactions and can go reversibly from a stable network structure to polymers solution based on the changes in external stimuli [6].

1.1.2. Chemical Crosslinking Strategies

Hydrogels are hydrophilic in nature and retain an insoluble matrix structure that originates from the crosslinks. The number of crosslinking points or degree of crosslinking is a major factor in determination of hydrogel's mechanical properties; as usually stronger and tighter hydrogels can be prepared by increasing the degree of crosslinking. Although, by increasing the crosslinking degree more brittle hydrogels are prepared; the demand is still to achieve elastic hydrogels with a pronounced mechanical strength.

Fabrication of hydrogels through chemical crosslinking is often preferred when long stability and high mechanical strength are demanded. In this method, permanent covalent bonds between monomer or polymer units are accomplished by utilizing various chemical reactions. A commonly employed chemical crosslinking strategy is free radical crosslinking of vinyl monomers with divinyl crosslinkers. In free radical crosslinking,

hydrogel formation is usually conducted by thermal polymerization, photo polymerization, radiation polymerization and plasma polymerization [7]. Other covalent strategies include enzyme mediated polymerization [8], click-chemistry based methodologies [9, 10], Diels-Alder [11, 12] and Michael addition reactions [13, 14].

Injectable hydrogels or in situ gelling systems exhibiting sol-gel phase transitions in response to change in stimuli can be prepared by utilizing some of above-mentioned covalent crosslinking methods. Michael addition-type reactions and photopolymerization can be considered as suitable methods for in situ gelation due to their fast reaction kinetics [15]. Dual cross-linked hydrogels comprising initial physical and post-chemical gelation can also be accomplished using several covalent bonding strategies [7].

Among the various available covalent bonding strategies, metal-free conjugation strategies are attractive for fabrication of hydrogels intended for biological applications since residual metal impurities can lead to loss of activity or deactivation of the biological entity. Specifically, addition reactions between thiol and alkene functional groups under radical-initiated conditions and the thiol-maleimide Michael addition reactions have been proven as versatile methodologies for preparation of cross-linked networks [16]. Using the photochemically induced radical thiol-ene reaction, spatial and temporal regulation during network formation can be achieved. Michael type nucleophilic thiol-ene reactions provide selective and fast conjugation between complementary functional groups. Both reactions can also proceed in the presence of air or in aqueous environment thus providing powerful techniques for fabrication of hydrogels for biomedical applications.

1.1.3. Thiol-ene Click Reactions

The reactions of thiols with alkene groups either proceeding by a radical initiated mechanism (radical thiol-ene reaction) or anionic chain initiation (nucleophilic thiol-ene reaction) are important sets of chemical transformations that are commonly referred as thiol-ene click reactions. Thiol-ene reactions form carbon-sulphur bonds while carrying many of the characteristics of click reactions such as near quantitative yields, rapid reaction rates, tolerance to the presence of air, oxygen and water and furnishing single regioselective products [17].

Radical thiol-ene reactions are implemented between thiols and alkenes, initiated by the formation of thiyl radicals and usually with the aid of UV light or thermal radical sources (Figure 1.2). The reaction is a typical addition reaction and the mode of the addition is anti-Markovnikov. The formation of thiyl radicals are usually accomplished by using photoinitiators (i.e. DMPA) or thermal initiators (i.e. AIBN).

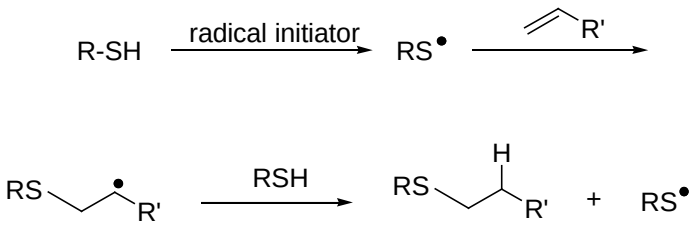


Figure 1.2. General mechanism of radical thiol-ene reaction.

The radical thiol-ene reaction can proceed in the presence of air or in aqueous environment thus providing a powerful technique for fabrication of various macromolecular platforms for biomedical applications. Combining the prominent advantages of click reactions, radical thiol-ene reaction has been shown as an efficient methodology to modify polymers, surfaces, synthesis of cross-linked networks, bio-conjugation and bio-immobilization of biologically active substances on polymeric materials [18].

A special type of thiol-ene reaction is nucleophilic thiol-ene Michael addition reaction that takes place between electron-deficient carbon-carbon double bonds and thiols. The reaction is based on a nucleophilic attack of thiol across an electron poor alkene, preferably α,β-unsaturated carbonyl compounds (Figure 1.3). The ene is usually chosen from (meth)acrylates, maleimides, fumarate esters and acrylonitrile. The reaction of thiols with maleimides is an outstanding example of metal-free click reactions that proceeds with excellent efficiency and rapid kinetics [17]. Due to the selective nature, high conjugation efficiency and benign reaction conditions, thiol-Maleimide click reactions have found immense application area in polymer synthesis, polymer functionalization, bioconjugation and related studies [19–21].

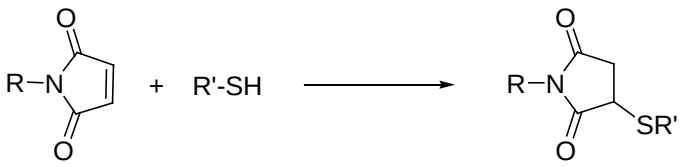


Figure 1.3. Representation of nucleophilic thiol-ene Michael addition of thiols and maleimides.

1.1.4. Thiol-ene Click Reactions in Hydrogel Fabrication

The addition of thiols to alkenes to form a thioether bond constitutes an important reaction in biosynthetic pathways as well as in chemical synthesis. Highly efficient nature of thiol-ene reactions have also been exemplified in design and synthesis of diverse macromolecular platforms including hydrogel networks. Due to the presence of thiol groups in many biomolecules, synthesis and modification of thiol-reactive hydrogel-based biomaterials offer ease of formulation and implementation in various applications.

Reaction of a thiol group with alkene functionality under radically-initiated conditions i.e. the radical thiol-ene click reaction has been shown as a useful methodology for fabrication and functionalization of hydrogel networks [22–26]. Hydrogels prepared by radical thiol-ene click reactions have been fabricated with a variety of cytocompatible polymers including poly(ethylene glycol) (PEG) [27], hyaluronic acid [28], and PEG-poly(lactic acid) copolymer [29], and modified peptide RGDS [26]. In a recent study, Anseth and co-workers demonstrated the utilization of radical thiol-ene click reaction on synthesis of hydrogels via thiol-norbornene photoaddition [30]. Four-armed PEG polymers bearing norbornene chain end units were reacted with dithiol-functionalized peptide linkers under UV light and using a photoinitiator (Figure 1.4). In a subsequent study, these hydrogels were conjugated with thiol-containing peptides with spatial and temporal control [26].

Photochemical thiol-ene-based crosslinking was elaborated by Ortiz and co-workers to fabricate sucrose-containing hydrogels [31] (Figure 1.5). In this study, diallylether functionalized sucrose molecules were treated with a multifunctional thiol containing crosslinker under UV illumination. The photoaddition reactions initiated by 2,2-

dimethoxy-2-phenylacetophenone (DMPA) were shown to result the cross-linked networks in less than 20 second and nearly 90 % thiol and ally functional group consumption.

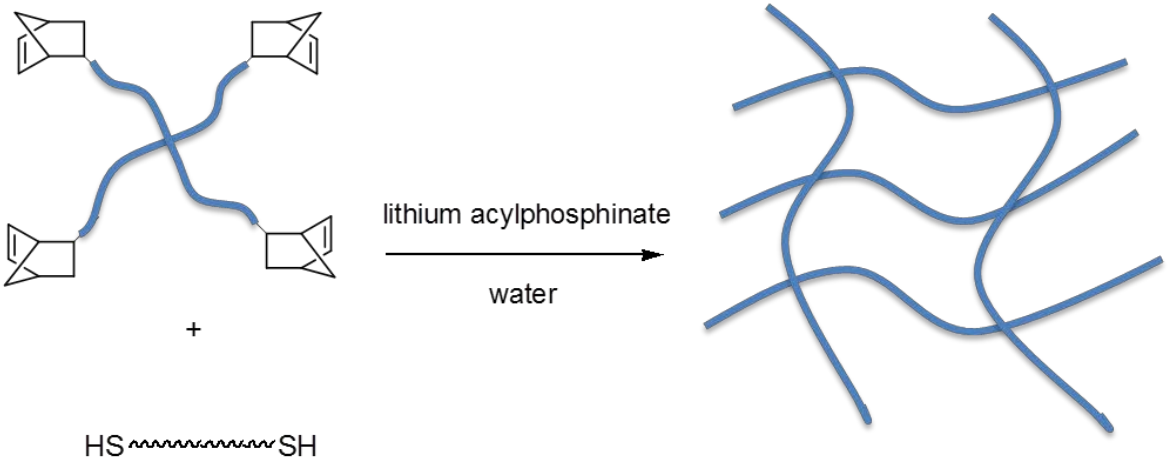


Figure 1.4. General scheme depicting the hydrogel network formation via radical thiol-ene reaction.

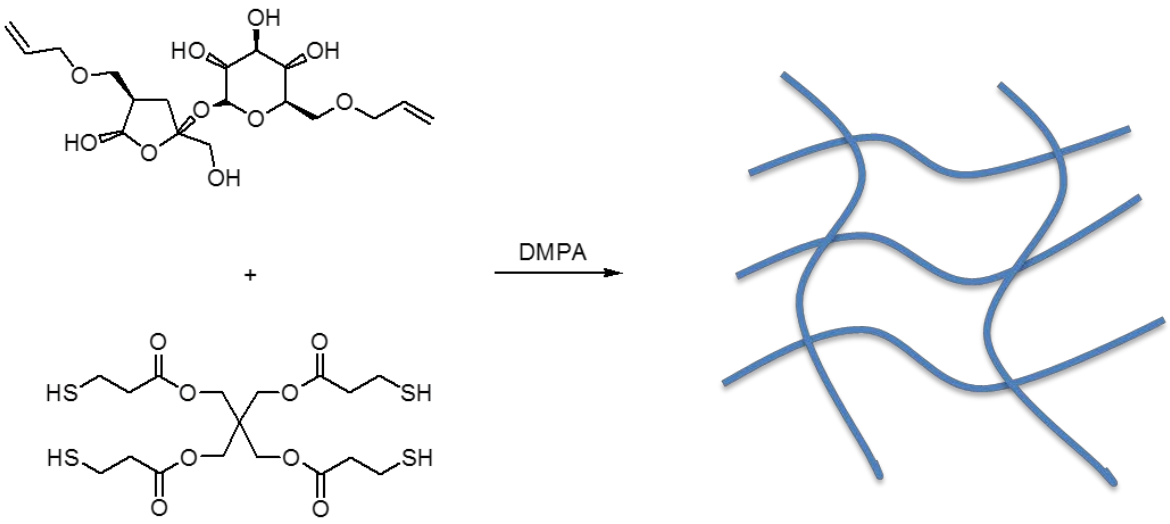


Figure 1.5. Radical thiol-ene conjugation of allyl ether-functionalized sucrose and tetra thiol-functionalized crosslinker to form hydrogel.

The addition reaction of thiols over electron deficient alkene groups is a special type of thiol-ene reaction known as the thiol-Michael addition click reaction. Maleimide, as an

electron deficient ene with outstanding reactivity towards thiols has a significant role in design of polymeric platforms based on Michael addition reaction. As highlighted by many literature examples, maleimide-thiol addition reaction is a highly valuable tool in click chemistry toolbox for fabrication and functionalization of hydrogels [32–35].

Recently, Prestwich and co-workers explored the preparation of hyaluronic (HA) based hydrogel via utilizing thiol-maleimide addition reaction [34]. Extracellular matrix (ECM) mimicking hydrogels that can be used for three-dimensional cell culture studies were obtained by crosslinking of thiol-modified derivatives of hyaluronic acid with maleimide homo-bifunctional PEG derivatives (Figure 1.6).

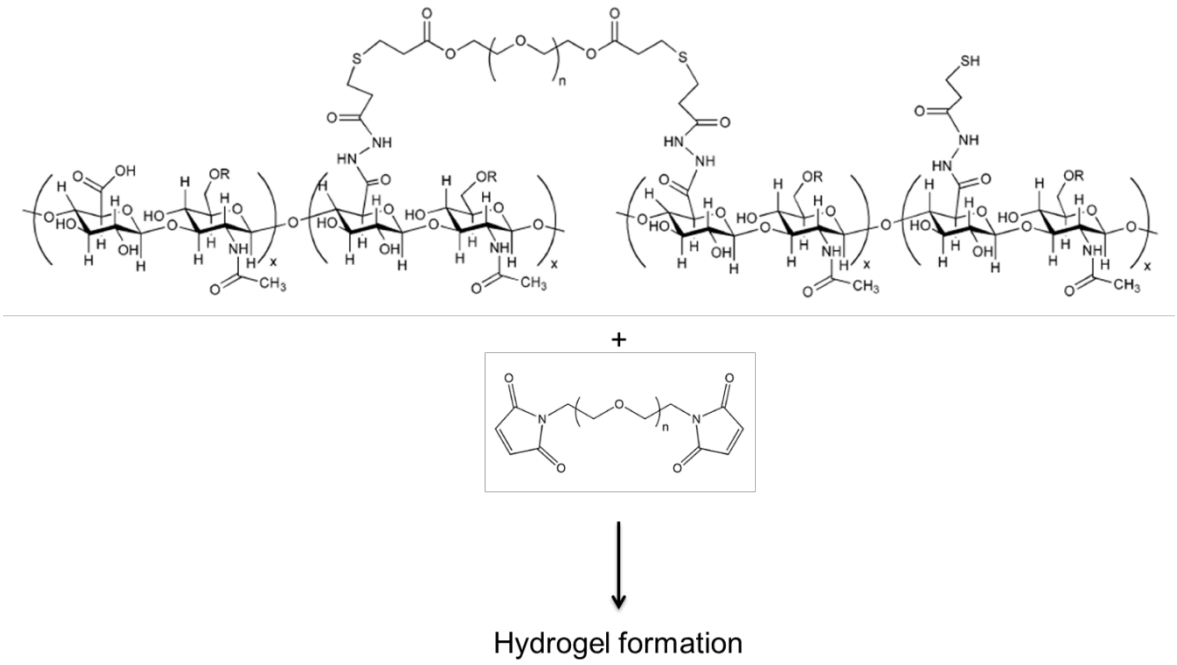


Figure 1.6. Preparation of hyaluronic acid-based hydrogels via thiol-maleimide addition reaction.

1.1.5. Hydrogels as Biomaterials

Hydrogels have been investigated extensively as an important branch of macromolecular chemistry and due to many unique properties, they have been considered as indispensable materials for various applications. Important characteristics of hydrogels as biocompatibility, tunable water uptake and physical characteristics similar to soft tissues make hydrogels attractive materials for various biomedical applications. Hydrogel based

macromolecular platforms have been intensely explored for fabricating controlled release systems for drug delivery, platforms for biomolecular immobilization, implant coatings, contact lenses and wound healing dressings [36–38]. Their structural similarities to the natural extracellular matrix enables mimicking natural systems for tissue engineering [34]. Biologically relevant molecules such as peptides and growth factors are incorporated onto structural scaffolds to interface with biological materials in a desired fashion *e.g.* promote cellular functions to enable tissue formation [39, 40]. In many cases, the aforementioned applications necessitates the presence of reactive units in gel network that can be functionalized with small molecules such as drugs and peptides or large molecules such as proteins and growth factors. Efficient post-polymerization functionalization of hydrogels thus has utmost importance to impart the desired functional attributes to these materials. The past decade has witnessed an immense increase in the adaptation of post-polymerization functionalization strategies to decorate hydrogel networks [41, 42].

1.1.6. Poly(ethylene glycol) (PEG)-Based Hydrogels as Suitable Materials in Biomedical Applications

It is well appreciated that hydrophilic polymers provide distinct advantages in synthesis and applications of hydrogel-based materials. Hydrogels with many potential applications often utilize poly(ethylene glycol) (PEG)-based systems [35, 43]. Poly(ethylene glycol)s have important characteristics in biomaterial applications as exhibiting rapid clearance from the body and resisting to recognition by the immune system [44]. PEGs are also well-studied polymers and have been approved in terms of extensive range of pharmaceutical applications by authorities like FDA. PEG is a non-toxic compound and PEG-based formulations can be injected direct into the body without causing adverse effects. PEGylation of biological agents has emerged as an important technique. The attachment of PEG derivatives to drugs, peptides or proteins have shown to improve their pharmacokinetic properties. In summary, due to the desirable characteristics such as antibiofouling, biocompatibility, rapid clearance from the body, non-toxicity and resistance towards recognition by the immune system, PEG-based polymeric materials have been extensively explored in biological and pharmaceutical applications.

1.1.7. Functionalizable Hydrogels

Functional or functionalizable hydrogels carrying chemically bound reactive groups that can undergo post-gelation modification reactions are remarkably versatile macromolecular platforms that find variety of applications in different areas [30, 41, 45]. The large interest of designing new hydrogels that allow post-polymerization functionalization stem from the possibility of hydrogel modification, attaching desired reagents onto these hydrogels or constructing other hydrogel based materials by utilizing the existing reactive groups. Hydrogel-based scaffolds bearing strategically placed reactive groups are thus suited in material engineering and biomedical-pharmaceutical sciences to selectively bind chemical agents or to tune and modify the hydrogel properties. Functional hydrogels contain polymer segments carrying reactive functional groups that enable the attachment of various compounds via covalent reactions. Commonly employed functional groups include activated esters, click chemistry components, epoxides, amines, aldehydes and ketones. Advances in design of efficient chemical transformations and new synthetic methodologies allow one to fabricate precisely functionalized hydrogels. These types of hydrogels find applications in wide range of areas such as biomolecular immobilization, drug delivery applications, tissue engineering and biosensor fabrication [46, 47].

For the conjugation of biologically relevant molecules to hydrogels commonly referred as immobilization, a much preferred functionalization strategy is based on the use of thiol groups. Thiols are present on many biomolecules or they can be incorporated as cysteine residues at desired positions. Thiols of cysteine groups in biomolecules have been shown to undergo efficient reactions with maleimides [48, 49], disulphide containing molecules [50, 51] and vinyl sulfones [52]. These functional groups are incorporated into hydrogels to offer a handle for obtaining polymer based bioconjugates.

The maleimide functional group has been extensively utilized for covalent immobilization of biomolecules. It has a significant role in both fabrication and functionalization of hydrogels. Due to the orthogonal nature to a wide variety of functional groups, maleimides are utilized in bioconjugation experiments requiring fast, efficient, quantitative and reagent free reaction conditions [53]. Maleimide is a substrate in reaction

with thiols in thiol-ene reactions, with dienes in Diels-Alder reactions. Maleimide can also undergo free radical polymerization due to the presence of activated alkene group.

Recently, Sanyal and co-workers demonstrated the fabrication of poly(ethylene glycol)-based hydrogels containing reactive maleimide functionalities [20]. Hydrogels were obtained by free radical polymerization of a PEG-based monomer (PEGMEMA) with a furan-protected maleimide-containing monomer (Figure 1.7). During the polymerization, some of the protected-maleimide groups were shown to undergo deprotection, resulting the crosslinking of hydrogel. The remaining maleimide groups were also recovered by heating the polymers at elevated temperature. The quantity of maleimide groups in the hydrogels were tailored by changing the feed ratio of used monomers during gelation. The obtained reactive hydrogels were efficiently functionalized with thiol containing molecules via the thiol-maleimide addition reaction.

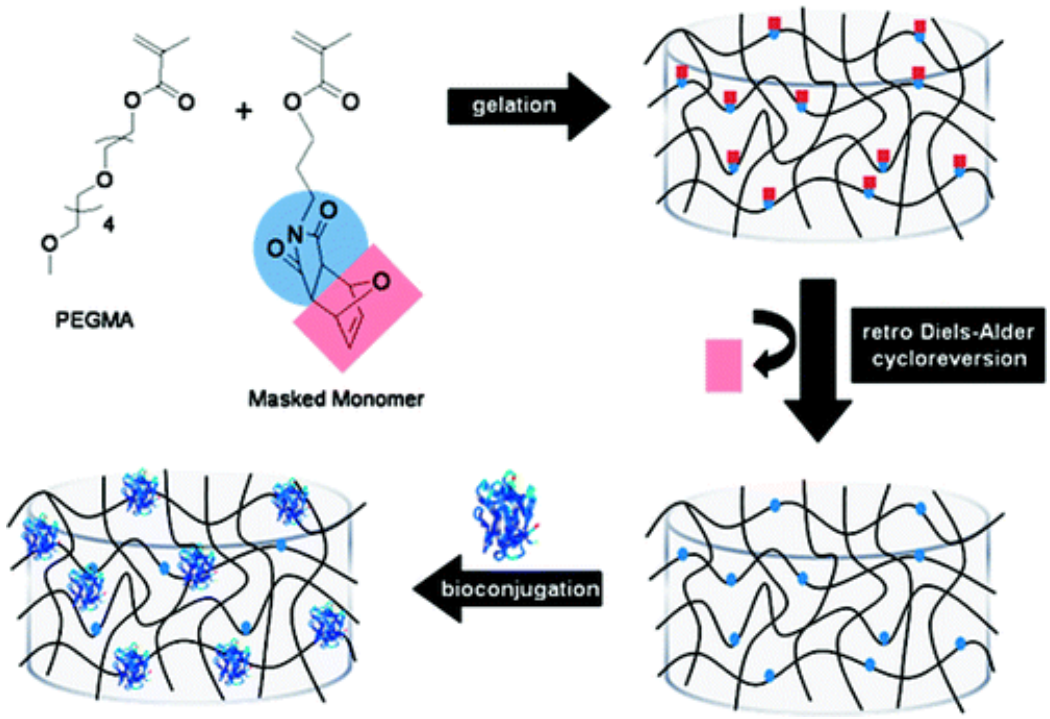


Figure 1.7. General scheme of fabrication of maleimide containing hydrogel using a latent reactive monomer (From [20] with permission).

By combining efficient design, fabrication and modification strategies, in the cross-line of chemistry and biology, thiol-based coupling strategies provide valuable tools in

generation of novel polymeric materials. Tsai, Jiang and co-workers recently reported the preparation of degradable poly(carboxy betaine) (pCB) hydrogels via thiol-disulfide exchange reaction [54]. Hydrogels were obtained by reacting a dithiol functionalized pCB with pyridyl disulphide containing CB copolymers (Figure 1.8). In order to evaluate the hydrogels in cell encapsulation studies, a growth factor, RGD peptide was successfully incorporated into hydrogels using thiol group of cysteine residue on the peptide molecule.

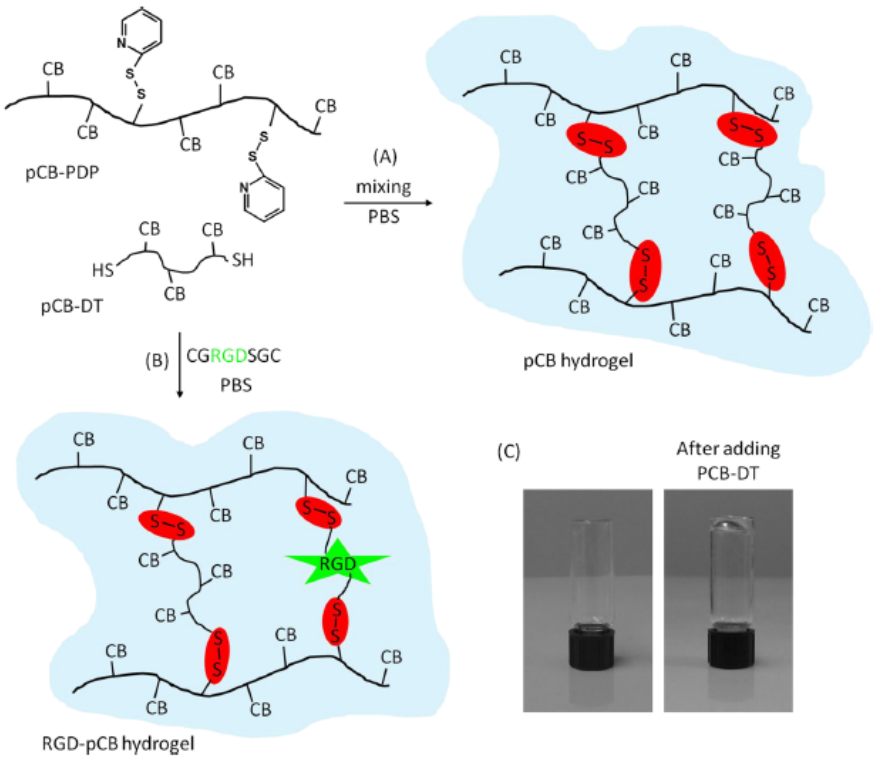


Figure 1.8. Schematic illustration of fabrication and functionalization of disulphide-containing hydrogels (From [54] with permission).

1.1.8. Hydrogels in Controlled Drug Delivery Applications

As mentioned in previous sections, hydrogels are attractive polymeric materials for extensive range of biomedical applications since they have porous structures with high water absorbing capacity and their structural similarities to the natural extracellular matrix (ECM) enables the mimicking of natural systems. The ability of swelling while preserving the viscoelastic properties, flexibility, high biocompatibility and tunable mechanical properties make hydrogels suitable materials for use. An important application of

hydrogels includes the fabrication of drug delivery systems which have attracted much attention during a few decades [55, 56].

Design of controlled drug delivery systems stem from the need of delivering or releasing the drugs in a controlled manner mainly at a desired rate and period of time. So the main purpose of a controlled drug delivery system is releasing the drugs in conformance with the aimed therapeutic effect.

Drug delivery systems usually divided into two general categories: ‘Targeted drug delivery systems’, systems delivering pharmaceutical agents to the specific part of body, organs or tissues and the ‘controlled drug delivery systems’, allowing the release of active agents at a predetermined rate and predefined time period. The interest of hydrogel-based polymeric platforms for rate or time-controlled drug delivery is continuously rising. These systems are designed to function as reservoirs that load the drugs and release them in a sustained way. Controlled drug release systems have been thought to have more potential effectiveness over traditional medication by eliminating under and overdosing, sustaining drug levels in a desired range, lowering the drug administration times and increasing patient compliance [57].

The aim of a controlled release system is to provide a sustained release of the drug in order to attain required drug concentration in blood or body fluids, over an extended period of time. In case of traditional drug administration, after dosing, drug level in blood suddenly increases and gradually decreases to low levels till the next administration (Figure 1.9). Usually, right after the dosing of drug, drug concentration reaches toxic overdosing levels and by time, falls down to below of minimum desired level. The primary effect of a controlled or sustained drug delivery system is to maintain the blood drug concentration in between the maximum and minimum levels for a prolonged time period [58].

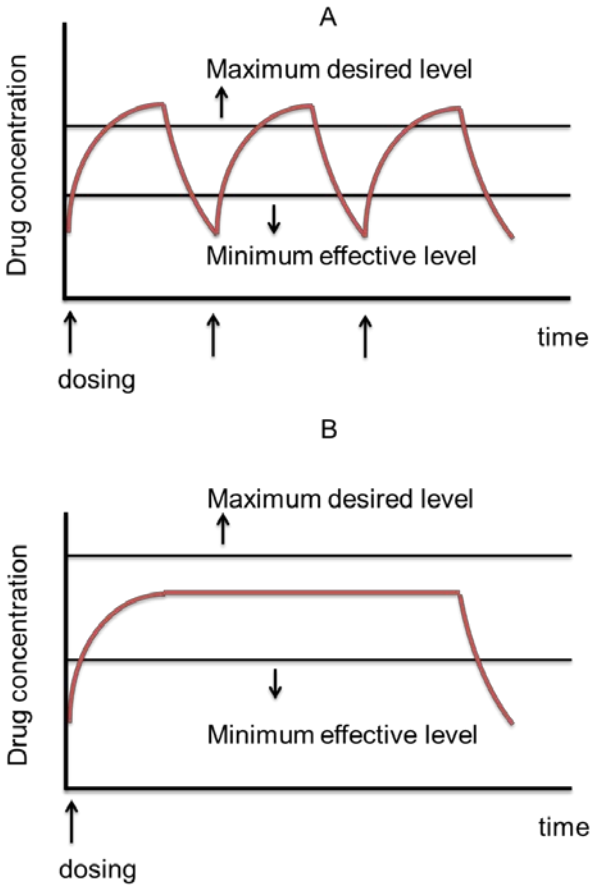


Figure 1.9. Comparison of plasma drug levels in a) traditional drug administration and b) controlled drug release administration.

Fabrication of hydrogels for effective drug loading and controlled release profiles requires efficient methods for their functionalization with molecules of interest. The loading of drug molecules to hydrogels can be achieved by either covalent or non-covalent attachment of drug molecules. Although covalent attachment of drug molecules offers advantages such as control over the release process by designing appropriate cleavable linkers to achieve stimuli–responsive release, oftentimes the process requires additional synthetic steps and complex formulations. On the other hand, functionalization of hydrogels with molecules of interest using non-covalent encapsulation is operationally simple and straightforward. Generally, loading of drug molecules within the hydrogel matrix are based on weak interactions, such as electrostatic, hydrogen-bonding, or hydrophobic interactions. The release profile of the drugs depend on parameters such as initial drug content, chemical environment of the encapsulated molecule, macroscopic factors such as thickness and degradability of the hydrogel, characteristics of the release

media, a few of the many factors that affect diffusion processes. Although a lot of attention has been focused on the utilization of hydrophobic interactions for sustained drug release, the process suffers from poor selectivity and specific control over drug loading. The approach oftentimes necessitates design of hydrogels that incorporate hydrophobic domains, which may introduce undesirable characteristics such as changes in microstructure and swelling properties. Functionalization of the hydrogel matrix with molecules possessing well-defined hydrophobic pockets such as cyclodextrins that can bind molecules of interest with specificity thus offers a versatile and powerful approach for achieving efficient drug loading and release.

1.1.9. Cyclodextrin Containing Hydrogels

A wide variety of hydrogel-based platforms obtained from natural and synthetic polymers have been investigated for drug-delivery carriers. However, loading hydrophilic matrices of hydrogels with drugs possess several limitations. The main difficulty arises from the chemical structure of drug molecules and hydrogel. Hydrophobic or poorly water-soluble drugs usually have low interactions with hydrophilic hydrogels causing the low drug loading efficiencies while formulation. On the other hand, controlled or sustained release hardly maintained with hydrophilic drugs [59]. In order to overcome several limitations, a preferred strategy is to incorporate hydrophobic pockets into the gel matrix to govern the drug-carrier interactions. In this regard, hydrogels containing cyclodextrins have been investigated as promising candidates in many areas of drug delivery due to their ability to form inclusion complexes with various hydrophobic drug molecules in aqueous environments.

Cyclodextrins (CDs) are a family of torus shaped molecules that possess hydrophilic outer shell and a hydrophobic interior cavity built from cyclic oligosaccharides consisting of 1,4-linked D-glucopyranose units. This unique structure of CDs enables the incorporation of hydrophobic molecules into their inner cavity through inclusion complex formation in aqueous environment. The commonly utilized cyclodextrins contain glucose repeating units ranging from six to eight monomers in a ring structure (Figure 1.10). Cyclic oligosaccharides with 6, 7 or 8 repeating units are referred as α-CD, β-CD and γ-CD, respectively.

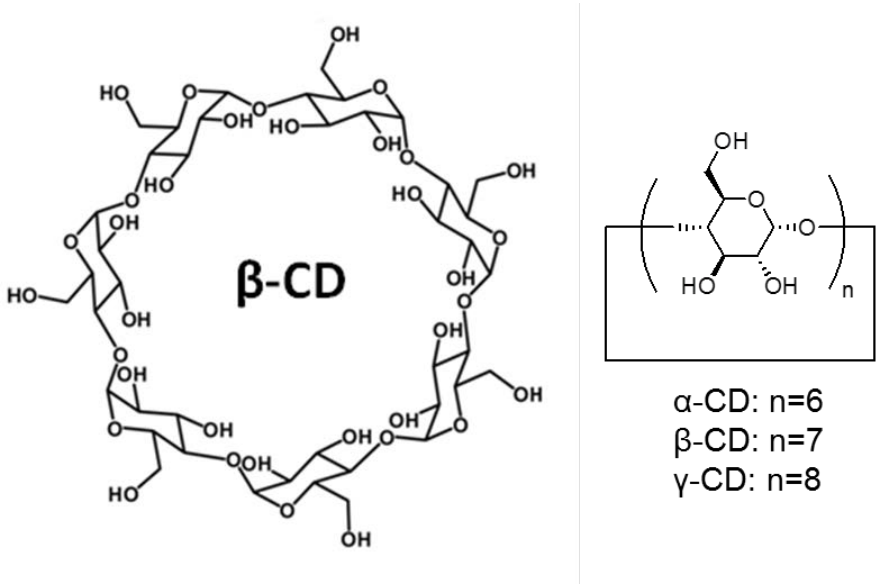


Figure 1.10. Chemical structure of the three main types of cyclodextrins.

The outer shell of cyclodextrins is very hydrophilic due to the presence of hydroxyl groups, while the inner region is quite hydrophobic. The hydrophilicity difference between inner and outer shells of cyclodextrins leads to a unique ability that they can form inclusion complexes with hydrophobic molecules in aqueous environments. Direct consequence of complex formation with hydrophobic molecules is to enhance the solubility and bioavailability of such compounds [60]. CDs have been extensively used in pharmaceutical sciences due to their ability to substantially increase solubility and bioavailability of inherently hydrophobic drug molecules [61, 62].

Hydrogels containing CDs have been investigated as promising candidates in many areas of drug delivery due to their ability to form inclusion complexes with various hydrophobic drug molecules in aqueous environments. The interaction of cyclodextrins with hydrophobic molecules is a dynamic equilibrium, rendering the formation and dissociation of inclusion complex dependent on environmental conditions [63]. In the presence of a competitive host molecule or dilution of the aqueous environment, the molecule included in the cavity can be released. Because of this behavior, cyclodextrins are extensively studied in the design of polymer/hydrogel-based sustained drug delivery systems to modify the release kinetics. In particular, for certain diseases where it may be difficult to maintain sustained concentration of drugs over prolonged periods such as in the

eye, localized delivery can be achieved using cross-linked polymeric materials such as contact lenses [64].

The ability of CDs to form inclusion complexes via specific host-guest interactions has been extensively exploited to obtain physically cross-linked hydrogels. This thesis does not include examples where the CD moiety plays the role of inducing physical networks but only covers examples where the CD molecules are covalently integrated into hydrogels.

The covalent incorporation of CDs to hydrogels can be managed by using several strategies. These techniques include (i) hydrogels synthesized using CD-based monomers; (ii) hydrogels using chemically modified CDs as crosslinkers; (iii) hydrogels obtained using unmodified CDs and (iv) hydrogels synthesized by using CD-containing polymers. In the following paragraphs, these methodologies are briefly discussed by giving literature examples as well imparting use of fabricated hydrogels in drug delivery applications.

Among the several methods of obtaining CD-containing hydrogels, one of the most commonly employed procedures involves the use of CDs as part of monomers, which can be incorporated into hydrogel network via free radical crosslinking [65–67]. Commonly used hydrophilic monomers for hydrogel fabrication include N-isopropylacrylamide, N-(hydroxymethyl) acrylamide, 2-hydroxyethyl methacrylate, methacryloyl modified hyaluronic acid, hydroxyethyl methacrylate, acryloyl modified poly(ethylene glycol), and acrylic acid. CD-containing monomers are generally obtained by modifying the primary hydroxyl groups on the lower ring of CD moiety using several acrylic-/methacrylic-based derivatizing agents. In a recent study, Zhang and coworkers designed a CD-containing monomer for the synthesis of hydrogels with fast shrinking kinetics [57]. Temperature-sensitive poly(N-isopropylacrylamide-*co*-acryloyl-β-CD) p(NIPAM-*co*-A-CD) hydrogels were prepared by polymerization of acryloyl-modified CD with NIPAM (Figure 1.11). Hydrogels were prepared in water or water/1,4-dioxane mixed solvent system using *N*,*N*′-methylenebis(acrylamide) crosslinker. The controlled drug release properties of poly(N-isopropylacrylamide-*co*-acryloyl-β-CD) hydrogels using ibuprofen (IBU) and tegafur (T-Fu) as model drugs were reported. From drug release studies conducted at 25 °C, in water, a prolonged release of ibuprofen was observed in case of β-CD incorporated hydrogels.

Higher drug loading was found for these hydrogels in contrast to pNIPAM hydrogels. This was attributed to inclusion complex formation between CD and ibuprofen. In case of the drug T-Fu, no such enhancements were observed, which indicated the lack of complex formation of the drug with CDs.

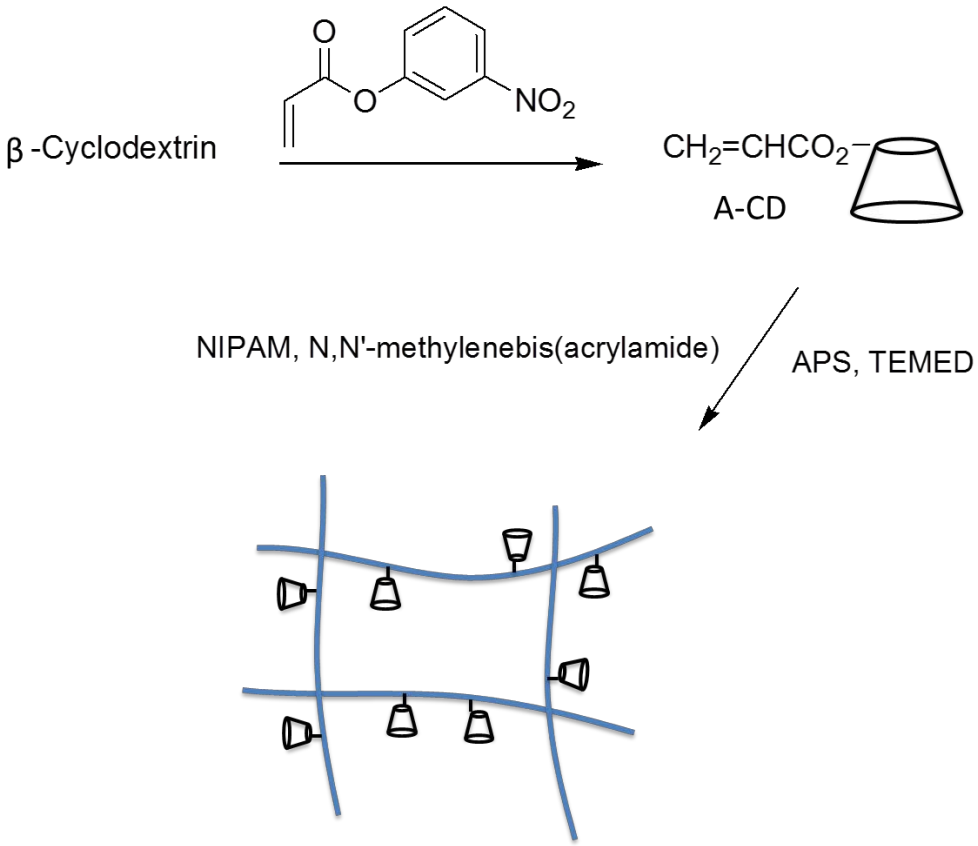


Figure 1.11. Synthesis of acryloyl-modified cyclodextrin and its use in hydrogel formation.

In the preparation of CD-containing hydrogels, many different covalent strategies have been successfully applied to incorporate CD moieties into the gel network. In addition to polymerization of CD-containing monomers, direct crosslinking using modified CD-based crosslinkers has also been employed toward the preparation of hydrogels [68, 69]. The multivalent chemical structure of CDs allows them to be used as multifunctional crosslinking reagents. Recently, Kros and co-workers demonstrated the preparation of dextran based hydrogels via efficient covalent coupling strategy between thiol and maleimide-containing compounds [70]. The hydrogel precursor of maleimide-functionalized dextran and thiol-functionalized β-cyclodextrin was covalently reacted to obtain macroporous networks (Figure 1.12). The obtained hydrogels were shown to be

potential biomaterials that can be used in control drug release applications of hydrophobic drugs.

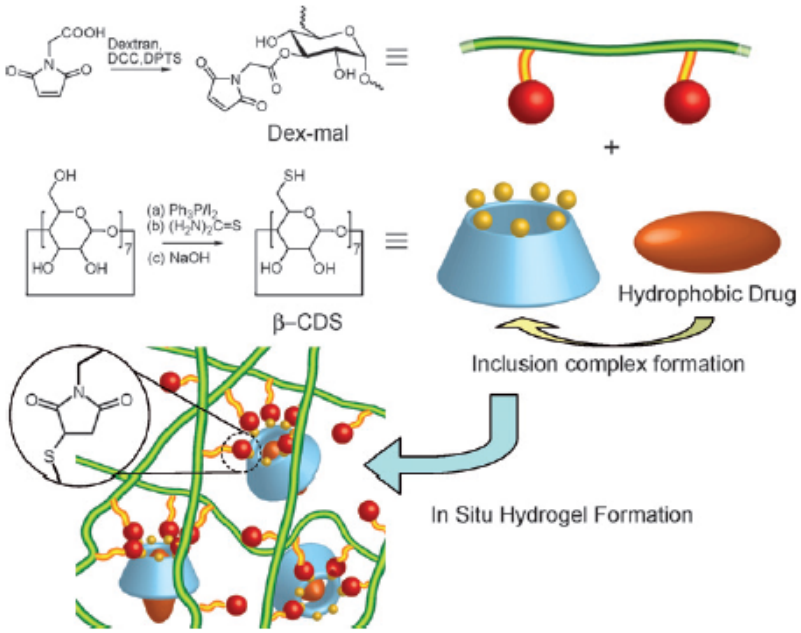


Figure 1.12. Schematic representation of hydrogel formation via thiol-functionalized β-CD (From [70] with permission).

With the aim of achieving hydrophilic polymeric networks containing chemically incorporated CD units, unmodified CDs have also been employed as crosslinkers. It is well known that primary hydroxyl groups are able to react with isocyanate, epoxide, and acyl functionalities and this strategy has been employed to form networks comprising CDs [59, 71, 72]. Although polymers carrying isocyanate and acyl functionalities have the ability of reacting CDs under mild conditions, generally reaction with epoxide groups require alkaline conditions. The chemical incorporation of native β-CD to acrylic hydrogels in a soft contact lens application was shown by dos Santos and coworkers [73]. Poly(hydroxyethyl methacrylate) (pHEMA) hydrogels were prepared by copolymerization with GMA at different compositions and β-CD was then grafted to the hydrogel network using the glycidyl groups. The obtained hydrogels were characterized in terms of the glass transition temperature, swelling degree, and viscoelasticity resulting that the attachment of pendant CD units did not alter the properties of the parent acrylic hydrogels. However, the ability of diclofenac loading was enhanced by 1300 % and the drug was sustained in lachrymal fluid for two weeks highlighting the advantageous effect of CD incorporation to the matrix.

Use of polymers carrying β-CD units along their backbone constitutes another important methodology for incorporating CDs into hydrogel matrices. Several synthetic routes of crosslinking CD-containing polymers to obtain hydrogels with desired properties are highlighted in this section. In a recent report, for instance, von Recum and co-workers reported the preparation of β-CD polymer and dextrose-based hydrogels for the delivery of antibiotics in a controlled manner [74]. Dextrose or the low-molecular weight water-soluble β-CD polymer (2-15 kDa) was chemically cross-linked with 2-isocyanatoethyl-2,6-diisocyanatohexanoate (LTI) or 1,6-diisocyanatohexane (HDI). The comparison of microstructures of hydrogels using SEM reveals that dextrose gel has smooth surface topology; whereas CD gels has broad network structures. Drug loading and release studies conducted with three different antibiotics (rifampin, novobiocin, and vancomycin) shows that CD-based gels have slower and more linear release rates compared with dextrose gels due to the inclusion complexation between CD groups and the drugs.

1.2. Functional Polymers

Functional or functionalizable polymers are macromolecules in which chemically reactive functional groups are bound to structure. The reactive groups present on the polymers allow the attachment of various chemical compounds onto the structure. The aim of polymer functionalization is based on imparting new properties (e.g. chemical, biochemical, physicochemical) to the designed materials [75]. The application areas of functional polymers may include the fabrication of solid supported catalysts, bioimmobilization and bioconjugation templates, optoelectronic applications (conducting polymers, magnetic polymers), dyes, paints, etc. [76].

The preparation of functional polymers can be achieved using different strategies. The first straightforward method includes the polymerization of functional group-containing monomers in which many functional polymers can be synthesized using radical, ionic polymerization or other polymerization techniques. Another strategy includes the synthesis of polymers that carry precisely positioned functional end group(s).

1.2.1. Synthesis of Functionalizable Polymers Using Reactive Functional Group-containing Monomers

The field of functional side chain polymers in polymer science has attracted great attention since these promising materials allow development of scaffolds enabling multivalent interactions. These side chains can be considered as ligands that can bind or interfere with certain groups especially in biological processes [77, 78]. In drug delivery applications, drugs, targeting groups or tacking agents can be loaded to polymers. The concentration of appended side chains allows the control of attached molecules.

Many functional groups including N-hydroxysuccinimide-based activated esters that allow the introduction of amine containing molecules, alkynes and azides that allow 'click' chemistries have been efficiently incorporated into polymers as pendant groups [79–82]. Another important functional group is maleimide that maleimides are excellent candidates for both Diels-Alder and Michael addition chemistries. In bioconjugation studies or immobilization of biomolecules, thiols play an important role. Peptides, proteins or enzymes containing thiols as cysteine residues can be selectively attached to polymers containing maleimide units. Apart from potential applications in biotechnology, polymers containing reactive maleimide units attracted considerable interest. Main difficulty in the synthesis of maleimide containing polymers is maleimide's itself since activated double bond of the maleimide also joins the polymerization. In many applications this drawback is eliminated by utilizing the versatility of the Diels-Alder reaction. The prominent advantage of Diels-Alder chemistry is its applicability mostly without the involvement of catalysts or coupling reagents. This is especially advantageous in terms of purification of materials; since in biological applications polymers are desired in high purity and without toxic metal residues. Another advantage is the thermoreversibility in which decomposition reaction (retro Diels Alder reaction) provides the initial reactants back. This property is well applied in the syntheses of polymers containing reactive maleimide groups. An initial masking of maleimide protects it during polymerization which masking groups are generally chosen from furan, fulvene or their derivatives. Removal of protecting groups by a retro-Diels-Alder reaction affords functional side chains.

The utilization of maleimide functionalized monomer for the synthesis of side chain functionalized polymer using free radical polymerization was demonstrated by Sanyal and co-workers [83]. A furan-protected maleimide containing methacrylate monomer was synthesized and used in AIBN initiated polymerization. The degree of maleimide functionalized monomer incorporation in the copolymer was tuned by changing the feed ratio of the monomer. After polymerization, thiol reactive maleimide groups were recovered by simply heating the polymers. In a later study, the monomer was used in the preparation of thiol-reactive polymeric micropatterns fabricated by thermal nanoimprint lithography (NIL) (Figure 1.13) [21]. The furan protected maleimide monomer was copolymerized with a poly(ethylene glycol)-based monomer and spin-coated onto a surface. During the NIL process, in situ deprotection occurs to furnish thiol-reactive polymeric micropatterns. The effective functionalization of thus obtained patterns was demonstrated by conjugating various thiol-containing molecules.

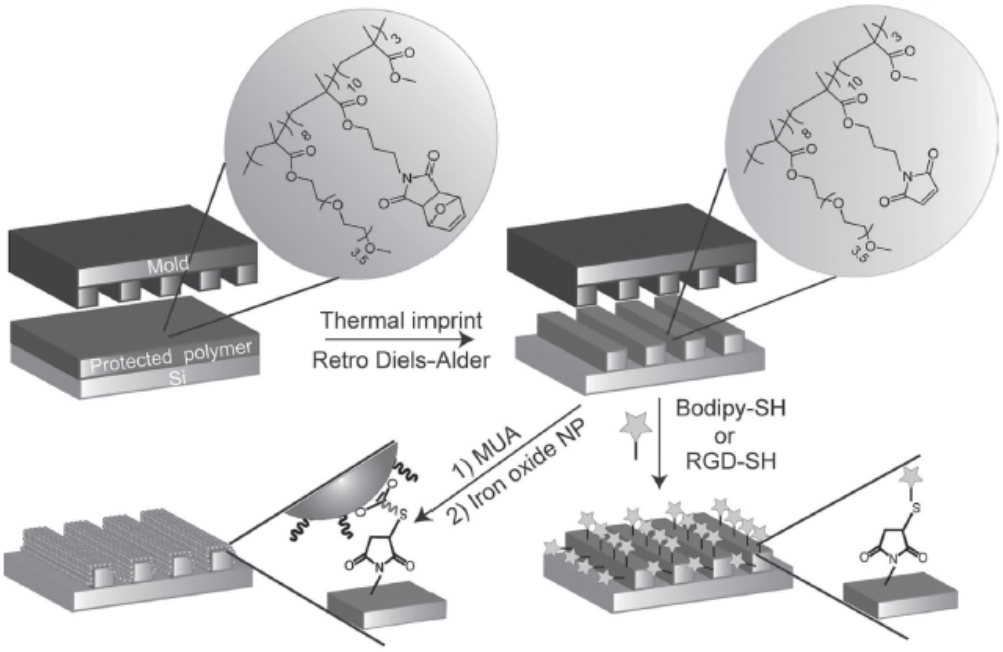


Figure 1.13. Synthesis and functionalization of maleimide containing polymeric patterns (Reproduced from [21] with permission).

Sanyal and co-workers have exploited the use of maleimide containing monomers to synthesize styrenic polymers [84]. A novel styrenic monomer containing protected-maleimide unit was prepared and used in the fabrication of both AIBN-initiated free radical polymers and RAFT polymers (Figure 1.14). Masked-maleimide groups residing at

the side chains were activated by removing protecting furan group under thermal conditions. To extend the methodology, maleimide containing monomer was copolymerized with N-hydroxysuccinimide-containing or alkene-containing styrene-based monomers to obtain orthogonally functionalizable styrenic types polymers. Sequential click reactions was successfully employed for the post-polymerization functionalization of polymers with model compounds of either amine-thiol or thiol-thiol functional group pairs.

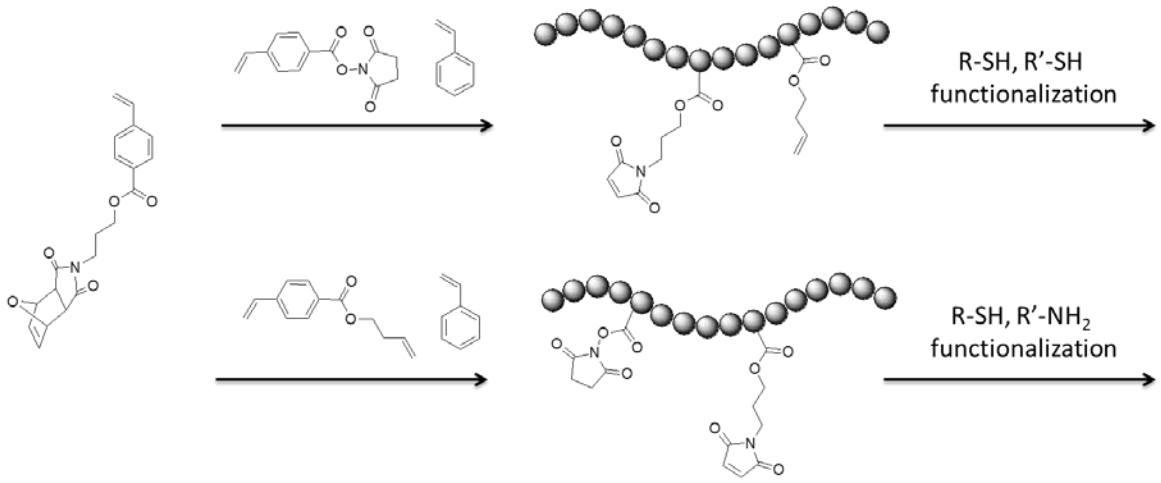


Figure 1.14. Synthesis and orthogonal functionalization of side-chain maleimide containing styrenic polymers.

1.2.2. End Group Functionalizable Polymers

Beside the strategy of incorporating functionalizable units onto polymer structures via polymerization of functional group containing monomers, another method includes the placing reactive groups at polymer chain ends. Taking the advantage of placing reactive groups as end group functionality, it is possible to achieve site-specific attachment of various accessories. Especially in designing macromolecules for biomolecule conjugation (such as proteins, peptides) it is important to achieve such specific modifications in terms of resulting properties [85]. Precisely appointed and well defined systems are also crucial for quantitative assessment of such conjugates.

Introducing functionality to the polymer end group can be accomplished using controlled polymerization techniques. Several initiators used in atom transfer radical polymerization (ATRP), nitroxide-mediated polymerization (NMP) and reversible-addition

fragmentation chain transfer (RAFT) have allowed to synthesize well defined polymers with reactive functional groups at polymer ends [86, 87].

Polymers containing one functional group at chain end are often referred as α-functional polymers and are used for (1:1) defined attachment of various chemical agents onto polymers. Polymer brushes fabricated through grafting polymer chains to surfaces are important applications of end functional polymers carrying one reactive chain termini [88]. Polymers bearing reactive functional groups at two chain-ends (α and the ω end groups) are known as telechelics and are important functional macromolecules used as functional supports, building blocks, crosslinkers and chain extenders [86].

Design and synthesis of new polymeric materials with specific properties is an important concept in the material science. A suitably fabricated polymer may itself function in an intended purpose; however preparation of nanocomposites by combining organic polymers with inorganic materials also increases the usefulness of the polymers. The leading properties of the polymers are inherently adapted to the new materials as well as gaining additional properties. A similar situation is also present in applications of polymers in biotechnology and medicine. Rationale design of polymers for bioconjugation of peptides, proteins and other biologically relevant macromolecules is a rapidly evolving area in the field. End functional polymers play an important role in fabrication of various polymer-biomolecule conjugates that find applications in biomedical fields. Polymer-peptide bioconjugates serve in drug delivery applications; as well they are suitable scaffolds for MRI contrast agents. Besides, attachment of bioavailable polymers such as poly(ethylene)glycols to proteins give rise to some additional benefits which are mainly due to the increased size of the proteins. The importance of synthesizing polymers which allow site-specific attachment of molecules is also apparent in terms of preparing human therapeutics.

The recent attention on peptide-polymer conjugates for their use in biotechnology and medicine has let the investigation of conjugation methods satisfying the needs. One of the important modification methods for site-specific conjugation is reacting rare amino acids or ligand binding sites with the proper ends of the polymers [89]. For such an aim, reactive cysteine residues provide an opportunity. Although in peptide structures not all

cysteines are in ‘free’ non-disulfide bond formed fashion, remedial free cysteine residues can be used for end group targeting. For proteins that not naturally contain free cysteines it is possible to place those using genetic engineering methodologies [90]. In choosing reactive end functionality of polymers to attach such amino acid residues, thiol reactive groups such as maleimides, vinyl sulfones and activated disulfides are mainly preferred [91]. Among these functional groups, because of high chemoselectivity and high reaction rate with thiol groups, maleimides are by far the most preferred reactive end groups in bioconjugate and biohybrid formulations. Syntheses of such polymers are generally performed by choosing a suitable initiator which allows the chemical attachment of the desired molecules. Since initiator starts the polymerization it is possible to have the same reactive functional group at the end of each polymer.

Haddleton and co-workers utilized the maleimide end functional polymers to fabricate glycopolymers enabling conjugation of thiol containing proteins [92]. The ATRP polymerization was conducted using the protected maleimide containing initiator and clickable alkyne groups were pendantly placed polymer backbone to introduce sugar units (Figure 1.15). In an alternative way sugar moiety was clicked to alkyne containing monomer and subsequently used for polymerization. After polymerization maleimide group was activated by removing masked furan moieties through a retro-Diels-Alder reaction. The specific thiol reactivity of the maleimide end functionalized glycopolymers were shown by attaching free cysteine residues of bovine serum albumin (BSA) and successful conjugation was confirmed by circular dichroism spectroscopy, SDS PAGE, and SEC HPLC analysis.

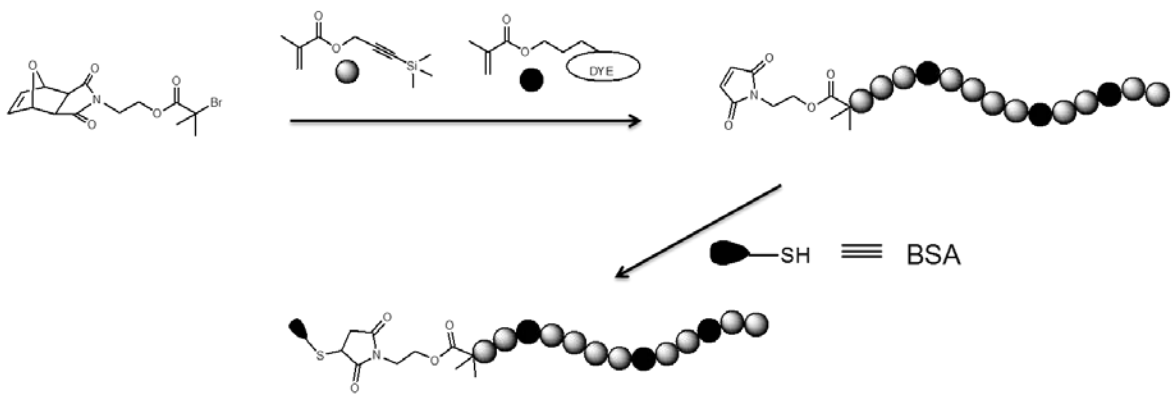


Figure 1.15. Synthesis of maleimide end functionalized copolymer and functionalization with BSA.

In atom transfer radical polymerization (ATRP), installing a functional group at the end of the polymer chains can easily be accomplished by choosing functional initiators. On the other hand, RAFT polymerization is another important technique to prepare end functional polymers with different topologies. Since ATRP polymerization requires the use of toxic metal catalyst, RAFT polymerization serves a good alternative for synthesizing polymers in a controlled manner without needing undesired toxic catalyst. In RAFT, introducing a functional group to polymer end is maintained by choosing a functional group containing chain transfer agent (CTA). To demonstrate this approach Maynard and co-workers investigated the synthesis of maleimide end functionalized polymers via RAFT polymerization for site-specific conjugation to free cysteines of proteins [93]. A trithiocarbonate chain transfer agent containing furan protected maleimide group was used to polymerization of poly(ethylene glycol) methyl ether acrylates (poly(PEGMEA)s) (Figure 1.16). After completing polymerization a subsequent retro-Diels-Alder reaction was performed to obtain thiol reactive maleimide groups at polymer ends. As post-functionalization of polymers, thiol-containing proteins were shown to efficiently bind to maleimide groups in a controlled manner.

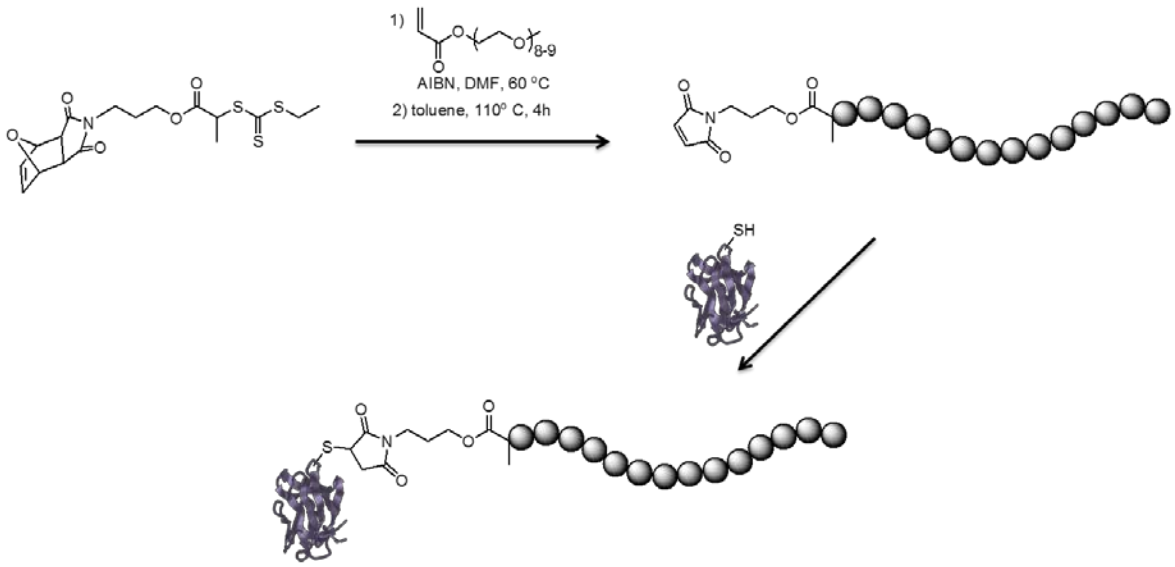
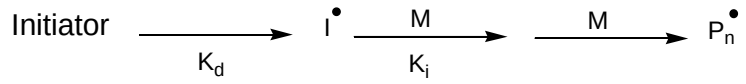


Figure 1.16. Synthesis of maleimide terminated polymers via RAFT polymerization and functionalization with protein.

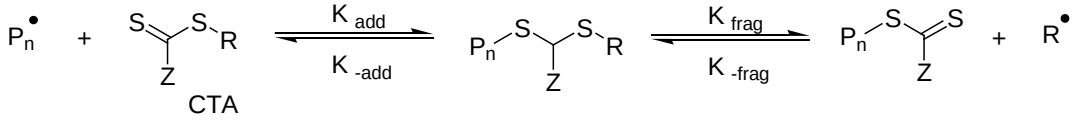
1.2.3. Reversible Addition Fragmentation Chain Transfer (RAFT) Polymerization

Reversible addition-fragmentation chain transfer polymerization is a controlled living radical polymerization technique along with the other methods such as, atom transfer radical polymerization (ATRP) and nitroxide-mediated polymerization (NMP), etc. RAFT method has been emerged as a powerful method for synthesis diverse polymeric architectures with low polydispersity indices and pre-determined molecular weights. The RAFT technique employs the use of dithiocarbonyl compounds to facilitate the polymerization via reversible chain transfer process. The mechanism of RAFT process allows an equilibrium in which propagating polymer chains grow evenly with respect to monomer conversion. The proposed mechanism of RAFT polymerization is depicted in Figure 1.17.

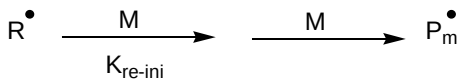
Initiation



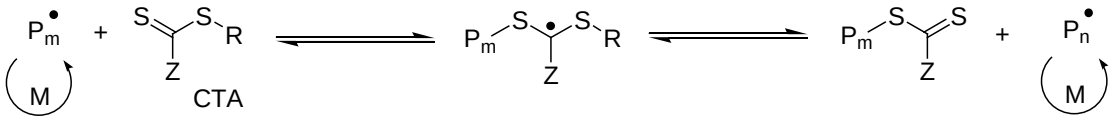
Chain Transfer



Re-initiation



Equilibrium between active and dormant chains



Termination

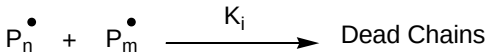


Figure 1.17. General mechanism of the RAFT polymerization.

In RAFT process, the activation and deactivation reactions form equilibria and are referred as chain-transfer reactions. Through the mechanism, active propagating chains convert from radical form to transfer agents allowing the proportional growth of the chains [94]. The method possesses important advantages such as, broad functional group and solvent tolerance, compatibility for vast range of monomers and devoid of requiring any toxic metal catalysts [95].

RAFT polymerization is a highly efficient technique to prepare well-defined end-functional polymers. The main component in the RAFT procedure is chain transfer agent (CTA), typically a thiocarbonylthio compound responsible for the controlled growth of the polymerization. The functional group present on the CTA (R group) is retained at the one chain end of the polymer enabling the synthesis of α-functional polymers. The RAFT group (thiocarbonylthio and Z group) can also be further manipulated thorough various methods to introduce desired functionality at ω-end. Figure 1.18 represents the utilization of RAFT process to obtain polymers with functional groups.

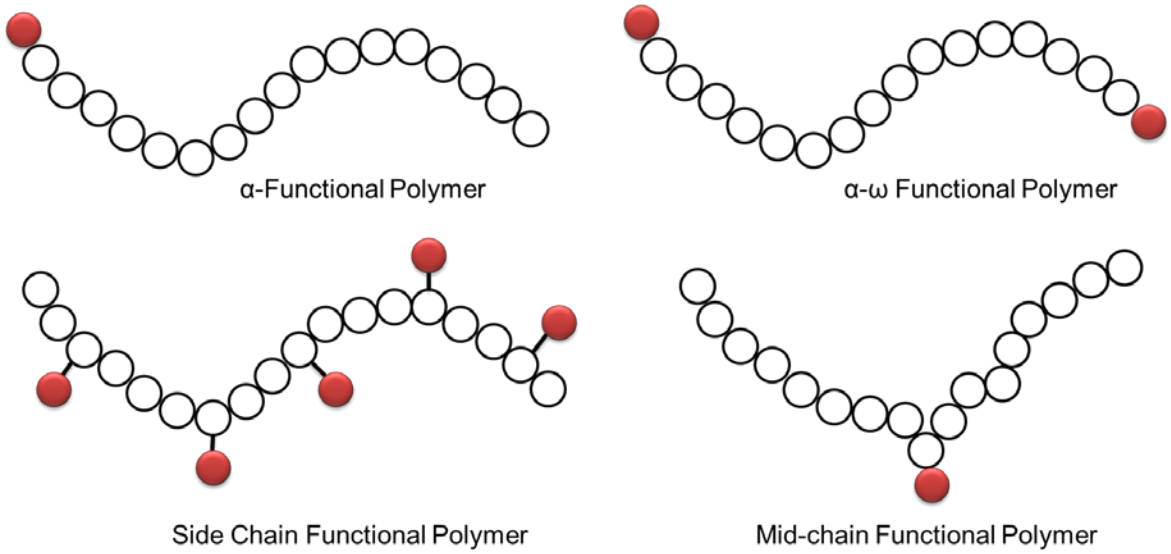


Figure 1.18. Overview of functional macromolecular architectures accessible via RAFT polymerization.

1.2.4. End Functional Polymers Through RAFT Polymerization

The RAFT process is convenient method to prepare end functional polymers. In a RAFT polymerization, monomers are inserted between the *R* group and the *Z* group of the chain transfer agent resulting the macromolecular structure of *R*(α)–polymer–CTA–*Z*(ω). As the general structure of CTA is retained in the synthesized polymer, the process serves a pronounced opportunity to prepare one end functional and two end functional (telechelic) polymers. Specific end groups can placed at α-end group by choosing functional group containing CTA's at *R* group. Several reactive end groups have been incorporated at polymer end chains using this strategy [96]. A broad range of applications might be driven by incorporating broad range of reactive groups at polymer ends.

An alternative strategy to incorporate functional groups to chain end in RAFT polymerization is based on the chemical modification of thiocarbonylthio end groups. Several methods including thermolysis, hydrolysis, aminolysis, oxidation-reduction and radical induced cross couplings have been used to incorporate various functional groups onto ω-ends of RAFT polymers [97] (Figure 1.19).

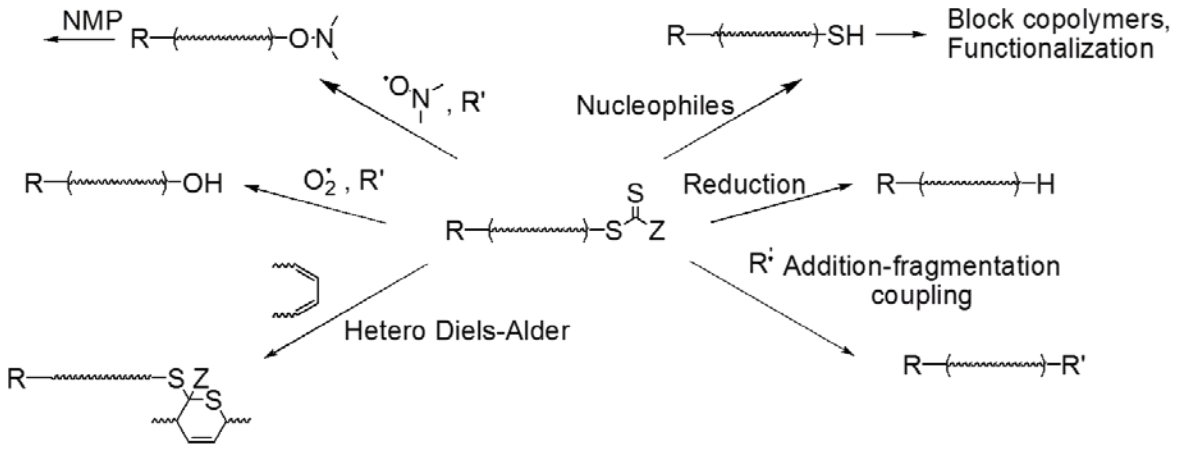


Figure 1.19. General overview of methodologies for RAFT end-group modification.

The subsequent chapters of this thesis will focus on fabrication of novel polymeric materials such as reactive polymers for drug delivery, hydrogels and polymeric coatings. The approach will utilize many of the aforementioned concepts and will aim towards design of materials at par beyond the current state of art in these areas.

2. RESEARCH OVERVIEW

The scope of this dissertation is to design, fabricate and investigate functional polymeric materials that can be suited for biomedical applications. Several methodologies were probed to synthesize functional polymers and hydrogels and these materials were evaluated in post-polymerization modification reactions to demonstrate their potential use. Functionalizable hydrogels, drug releasing hydrogels, micropatterned hydrogels, functionalizable polymers for post-polymerization modification reactions, surface grafted functional polymers and functional polymer-based drug delivery vehicles are main concepts that were discussed in this manuscript.

The first three projects in this dissertation are related to the fabrication of structurally well-defined hydrogels based on cyclodextrin mediated polymer crosslinking strategies. Hydrogels are an important class of cross-linked polymeric materials that play a crucial role in various biomedical applications, such as fabrication of drug delivery systems and scaffolds for tissue engineering. Encapsulation of various drug molecules via non-covalent functionalization of hydrogels has been widely used for achieving controlled release. Incorporation of cyclodextrins (CDs), a class of cyclic oligosaccharides, into hydrogels can enhance loading of certain hydrophobic drugs and allow their slow release. The first scope of research is to fabricate CD-containing hydrogels with well-defined network structures and their use in target molecule conjugation and controlled drug release studies.

The second scope of the research is to synthesize functional polymers that are amenable post-polymerization modification reactions. Functional polymers are important scaffolds that find applications in bioimmobilization and bioconjugation templates, fabrication of solid supported catalysts and optoelectronic applications. Functional polymers carrying end-group and side chain reactive functionalities were synthesized using step growth and controlled radical polymerization techniques and their use in post-polymerization modification were investigated.

3. CYCLODEXTRIN MEDIATED POLYMER COUPLING VIA THIOL-MALEIMIDE CONJUGATION TO ACCESS FUNCTIONALIZABLE HYDROGELS

The materials in this chapter have been adapted with modifications from the following article: Arslan M., T.N. Gevrek, A. Sanyal, R. Sanyal, *RSC Advances*., Vol. 4, pp. 57834–57841, 2014.

3.1. Introduction

Especially in last few decades, there have been large efforts for both preparation of diverse hydrogel-based polymeric materials and their functionalization with vast range of biological and chemical agents for various applications. The excellent biocompatibility, ability of absorbing large amount of water and rubbery state similar to soft body tissues make hydrogels applicable in designing controlled release systems for drug delivery, biomolecule immobilization, contact lens applications and wound healing dressings [1, 3, 47]. Since hydrogels have structural similarities to natural extracellular matrixes (ECMs), they are also used to mimic these natural systems in tissue engineering studies [98, 99]. Developing peptide-functionalized hydrogels for 3D cell culture applications is also another important interest in the field [39]. The extensive application areas of hydrogels in biomedical fields can be summarized as the construction of tissue engineering scaffolds and cell culture growing mediums, controlled / sustained-release drug delivery systems, contact lenses, implants and biosensors, wound and burn healing dressings, disposable diapers and reservoirs for topical drug delivery, rectal diagnosis and drug delivery.

In the course of research, rapid growth in synthetic method development has also shaped the approaches of new soft material fabrications. As outlined in the previous sections, many different covalent strategies including copper mediated “click” methodologies, Diels-Alder reactions, free radical crosslinking and UV-crosslinking are widely used to obtain various kinds of hydrogel systems. Although, depending on the purpose, all these strategies possess important applicabilities, demands in industrial and research settings necessitates hydrogel systems to be prepared with more controlled and

well-defined architectures [100]. Functionality and reactivity of hydrogels are also important parameters especially in biomolecular immobilization and cell culture studies. From the point of synthetic chemistry, high conversion yields, cheap and abundant starting materials and simple chemistries also crucial. Accordingly, efficient, robust and orthogonal approaches to obtain hydrogels is still in high demand.

One of the most common covalent coupling methods in fabrication of polymer-based materials includes thiol-maleimide Michael addition reactions. High reaction yields, high selectivity and catalyst free reaction conditions of this versatile chemistry have provided a considerably fascinating approach for engineering materials. The efficiency of thiol-maleimide Michael addition reactions have been exploited for example, to prepare linear polymers [101], functionalization of thiol-reactive telechelic and semitelechelic polymers [102], networks and composite materials [103]. Preparations of diverse hydrogel systems based on thiol-maleimide chemistry have also gained much interest in the course [34, 103].

Many small molecules, biologically important macromolecules, peptides and enzymes can be incorporated into hydrogel networks. In order to obtain biocompatible systems specific covalent coupling methods are often employed especially in pharmaceutical and medical use of hydrogels. Due to the high reactivity and selectivity towards thiol containing molecules, maleimide functionalized hydrogels are also prevalent scaffolds for biomolecule immobilization. Since in conventional approaches, incorporating biomolecules into hydrogels matrix heavily based on encapsulation and physiabsorption [104], utilizing mild thiol-maleimide reactions give the opportunity of both covalent immobilization and post-gelation functionalization.

Cyclodextrins (CDs) are cyclic oligosaccharides with six to eight anhydroglucopyranose units. They have a toroidal structure as the primary hydroxyl groups places at the narrow side and the secondary hydroxyl groups at the wide side. CDs are readily available compounds with low prices and high purities; as well they have well-defined, rigid structures. The inclusion complexation ability of CDs with various guest molecules makes them attractive for drug delivery systems. The chemical structure of cyclodextrins also allows them to be used as multifunctional crosslinking reagents. Primary hydroxyl groups of cyclodextrin can be transformed into different functionalities resulting

multi-functional agents (Figure 3.1). By utilizing the multivalent nature of cyclodextrins many cyclodextrin-containing hydrogel networks have been prepared [69, 105, 106].

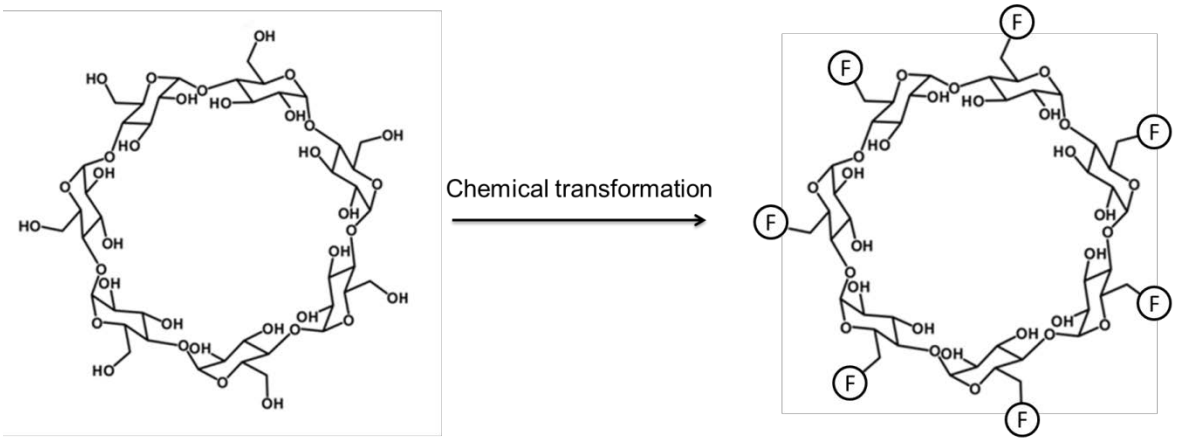


Figure 3.1. Chemical transformation of primary hydroxyl groups of a cyclodextrin into different functionalities.

The present study demonstrates the preparation of poly(ethylene glycol) (PEG)-based hydrogels chemically cross-linked via thiol-maleimide addition reactions (Figure 3.2) [107]. The synthetic approach employs maleimide end-functionalized poly(ethylene glycol)s as the backbone and thiol-functionalized β-cyclodextrin (β-CD) as crosslinker. High reaction rate between sulfhydryl group and maleimide functionality were utilized to prepare hydrogels. In order to allow post-gelation modification reactions enabling further introduction of functionality, crosslinker ratio were tailored. By changing the crosslinker ratio to adjust the stoichiometry, either thiol functionalized or maleimide functionalized hydrogels were prepared. Efficient functionalization of these reactive hydrogels in a tailored fashion was demonstrated through conjugation of appropriately functionalized fluorescent dye molecules.

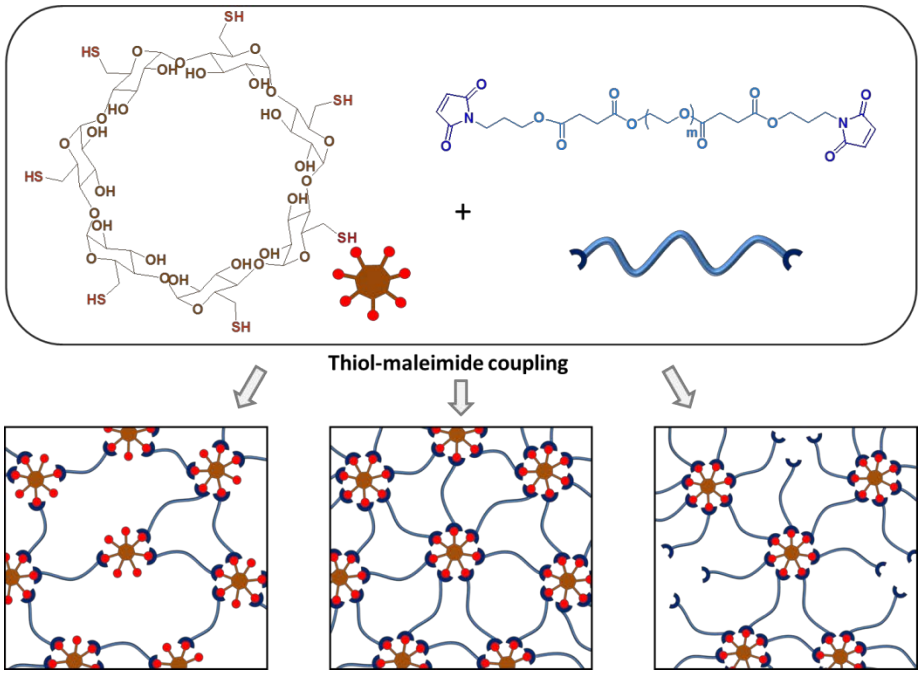


Figure 3.2. Schematic illustration of fabrication of hydrogels via thiol-maleimide conjugation.

3.2. Experimental

3.2.1. Materials and Characterization

Poly(ethylene glycol)s (PEG 2K, PEG 6K, PEG 10K), 4-dimethylaminopyridine (DMAP), (5,5'-dithio-*bis*-(2-nitrobenzoic acid), *N*-(5-fluorosceinyl)maleimide and fluoresceinamine, isomer-I were obtained from Aldrich Chemical Co. 1-Ethyl-3-(3-dimethylamino propyl) carbodiimide (EDCI) and β-cyclodextrin hydrate were obtained from Alfa Aesar. 5-((2-(and-3)-S-(acetylmercapto)succinoyl)amino)fluorescein (SAMSA fluorescein) dye was obtained from Invitrogen (Carlsbad, CA). Other chemicals and solvents were purchased from Merck and used as obtained without further purification unless otherwise noted. Synthesis of alcohol exo-3a,4,7,7a-tetrahydro-2-(3-hydroxypropyl)-4,7-epoxy-1H-isoindole-1,3(2H)-dione [108], thiol functionalized β-cyclodextrin [109] and PEG diacids [110], [111] were conducted according to reported procedures. Proton NMR spectral data were obtained using a Varian INOVA400 at 400 MHz. Thermogravimetric analyses (TGA) were carried out using a TA Instruments at a 10

^{o}C/min heating rate under nitrogen atmosphere. Dye functionalized hydrogels were visualized with Zeiss Observer.Z1 inverted fluorescent microscope.

3.2.2. Typical Synthesis of Bis-acid 2K PEG

Anhydrous PEG (2 g, 1 mmol) prepared by azeotropic evaporation of toluene was dissolved in THF (5 mL) and triethylamine (0.42 mL, 2.99 mmol) was added at 0 °C. Succinic anhydride (0.34 g, 3.42 mmol) and DMAP (0.05 g, 0.40 mmol) were dissolved in THF (5 mL) in a separate round bottom flask and the mixture was slowly added to the PEG solution at 0 °C in a drop wise fashion. The solution was stirred for 20 h under N_2. The reaction mixture was concentrated in vacuo, dissolved in minimal amount of CH_2Cl_2 and precipitated in cold diethyl ether twice to give pure bis-acid PEG as a white solid (2.13 g, 97 % yield) that was used directly in the next step. Similar protocol was used to obtain PEG-diacids from PEG-diols with different molecular weights.

3.2.3. Synthesis of Protected Bismaleimide PEGs

A flask equipped with a magnetic stirrer was charged with PEG 6K diacid (0.48 mmol, 3 g), protected maleimide containing alcohol (1.44 mmol, 320.0 mg), EDCI (1.05 mmol, 202.0 mg) and DMAP (0.096 mmol, 11.0 mg). A 10 mL portion of anhydrous CH_2Cl_2 was then added to reaction vessel and mixture was stirred overnight at room temperature under N_2 atmosphere. After reaction completed, more CH_2Cl_2 was added and mixture was extracted with 30 mL saturated $NaHCO_3$ solution. The organic layer collected and dried over Na_2SO_4. After filtering, solvent was removed under reduced pressure. Then, the polymer was dissolved in minimum amount of CH_2Cl_2 and precipitated by pouring into cold anhydrous ether. The precipitate was then filtered and dried in vacuo (71 % yield). ^{1}H NMR ($CDCl_3$, δ, ppm) 6.50 (s, 4H, CH=CH), 5.24 (s, 4H, C*H* bridgehead protons), 4.23 (t, 4H, *J* = 4.7 Hz, OCH_2), 4.04 (t, 4H, *J* = 5.8 Hz, NCH_2), 3.82-3.42 (m, OCH_2CH_2 of PEG), 2.83 (s, 4H, C*H*-C*H* bridge protons), 2.68-2.58 (m, 8H, CH_2CH_2C=O), 1.90 (tt, 4H, *J* = 5.8, 4.6 Hz, NCH_2CH_2CH_2O).

3.2.4. Activation of the Protected Maleimide Groups

Deprotection of maleimide groups were carried out by heating polymers at 110 ^{o}C in anhydrous toluene for 12 h. After completing the reaction, solvent was evaporated to

obtain the polymers in quantitative manner. ^{1}H NMR ($CDCl_3$, δ, ppm) 6.69 (s, 4H, C*H*=C*H*), 4.23 (t, 4H, *J* = 4.8 Hz, OCH_2), 4.06 (t, 2H, *J* = 5.7 Hz, NCH_2), 3.83-3.41 (m, OCH_2CH_2 of PEG), 2.67-2.57 (m, 8H, CH_2CH_2C=O), 1.93 (tt, 2H, *J* = 5.6, 4.7 Hz, $NCH_2CH_2CH_2O$).

3.2.5. Representative Hydrogel Formation

Hydrogels were prepared by multiple Michael type addition of thiol-functionalized β-cyclodextrin (β-CD$(SH)_7$) onto PEG-bismaleimide (PEG-*bm*) polymers. Typical procedure is as follows: The polymer (50 mg, 0.02 mmol for PEG 2K-*bm*) was placed in a vial and dissolved in DMF (100 µL). Desired amount of β-CD$(SH)_7$ and catalytic amount of triethylamine (1/20 eq. per thiol) were dissolved in DMF (100 µL) and then added to polymer solution. The stoichiometry of thiol to maleimide group was adjusted to obtain hydrogels with desired residual reactive functional groups. The mixture was sonicated to assist homogenous gelation. Hydrogel formation was rapid and in about one minute there was no flow of sample upon inversion of vial. Gelation was continued for 6 h to ensure complete conjugation. After hydrogel formation, unreacted species were removed by washing with DMF followed by distilled water. Swollen hydrogel sample was freeze-dried in vacuo to yield dried hydrogel.

3.2.6. Swelling Studies

Swelling studies were performed by immersing a known amount of hydrogel in distilled water and monitoring the increase in its mass as a function of time until equilibrium was reached. The percentage weight change was obtained from fractional weight change using the equation:

$$\text{Percentage of Swelling (\%)} = (W_{wet} - W_{dry}) / W_{dry} \times 100,$$

where W_{wet} and W_{dry} refer to the weight of wet and dry hydrogels respectively. All measurements were performed in triplicate and average data was plotted to obtain swelling curves.

3.2.7. Scanning Electron Microscopy (SEM)

The surface morphologies of hydrogels were analyzed with scanning electron microscopy (SEM). SEM images of dried hydrogels were acquired using an ESEM-

FEG/EDAX Philips XL-30 (Philips, Eindhoven, The Netherlands) instrument operating at an accelerating voltage of 10 kV.

3.2.8. Rheological Measurements

Hydrogel rheological behaviors were evaluated by measuring the loss (G″) and storage (G′) moduli of the swollen hydrogel as a function of angular frequency using an Anton Paar MCR 302 rheometer. Tests were carried out in triplicate at 25 ^{o}C by applying a 0.5 % strain between 0.05-100 rad/s. The samples prepared as disks were analyzed using a parallel plate (15 mm diameter) with a gap of 2.0 mm.

3.2.9. Determination of Sulfhydryl Content

Free sulfhydryl group content in hydrogels was determined using Ellman's method [112]. Firstly, a reaction medium of 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA buffer was prepared. Ellman's reagent solution was prepared by dissolving of 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) (4 mg/mL). A sample of freshly prepared hydrogel (5 mg) was placed in a vial and 2.5 mL of buffer and corresponding Ellman's reagent solution were added onto the solution. The resultant mixture was incubated at 37 °C for 2 hours. The absorbance at 412 nm was measured to calculate the total sulfhydryl group content in the sample using the molar extinction coefficient of 2-nitro-5-thiobenzoic acid (TNB) (14,150 $M^{-1}cm^{-1}$) [113].

3.2.10. Functionalization of Hydrogels with Maleimide-containing Fluorescent Dye

Functionalization of hydrogels containing free sulfhydryl groups were performed using *N*-(5-Fluorosceinyl)maleimide. First, the amount of free sulfhydryl groups in 5 mg hydrogel sample was calculated and ten equivalents of *N*-(5-fluorosceinyl)maleimide in PBS (pH 7.4, 0.5 mL) solution was added. After incubation at room temperature for 12 h, hydrogel was washed several times with organic solvents and deionized water before analysis of fluorescence microscopy. As a control experiment, a sample of $CDP6_{(2:1)}$ hydrogel was incubated with fluoresceinamine dye.

3.2.11. Functionalization of Hydrogels with Thiol-containing Fluorescent Dye

Hydrogels containing free maleimide groups were functionalized via thiol-containing SAMSA fluorescein dye conjugation. Firstly, protecting group of the dye was removed by treatment with 0.1 M NaOH at room temperature. Then, the solution was neutralized with concentrated HCl and buffered to pH 7.0 with 0.5 M sodium phosphate. The amount of free maleimide groups in 5 mg hydrogel sample was calculated and ten-fold excess of activated SAMSA fluorescein thiol solution was added. After incubation at room temperature for 12 hours, hydrogel was washed several times with organic solvents and deionized water before analysis using fluorescence microscopy. As a control experiment, a sample of $CDP6_{(1:2)}$ hydrogel was incubated with maleimide-functionalized fluorescein dye.

3.3. Results and Discussion

3.3.1. Synthesis of PEG-bismaleimides

Commercially available PEG-diols of varying molecular weights were appended with reactive maleimide functional groups at both ends. Briefly, the PEG-diols were converted to respective diacids by reacting with succinic anhydride, followed by coupling with a furan-protected maleimide containing alcohol. Removal of the furan protecting groups via the retro Diels-Alder reaction yields the maleimide-containing telechelic PEG polymers (Figure 3.3). The quantitative nature of this deprotection step was determined using ^{1}H NMR spectroscopy. Thermogravimetric analysis (TGA) of deprotected polymers shows that removal of furan group was achieved quantitatively (Figure 3.4). Thiol functionalized β-cyclodextrin crosslinker was synthesized by converting primary hydroxyl groups of β-CD molecule to thiol groups using a two-step procedure. The primary hydroxyl groups were first converted to iodo groups using iodine and triphenylphosphine, followed by their conversion to thiol functional groups by treatment with thiourea (Figure 3.5). Ellman's assay of thus obtained heptavalent CD-crosslinker revealed a thiol content of 98 %.

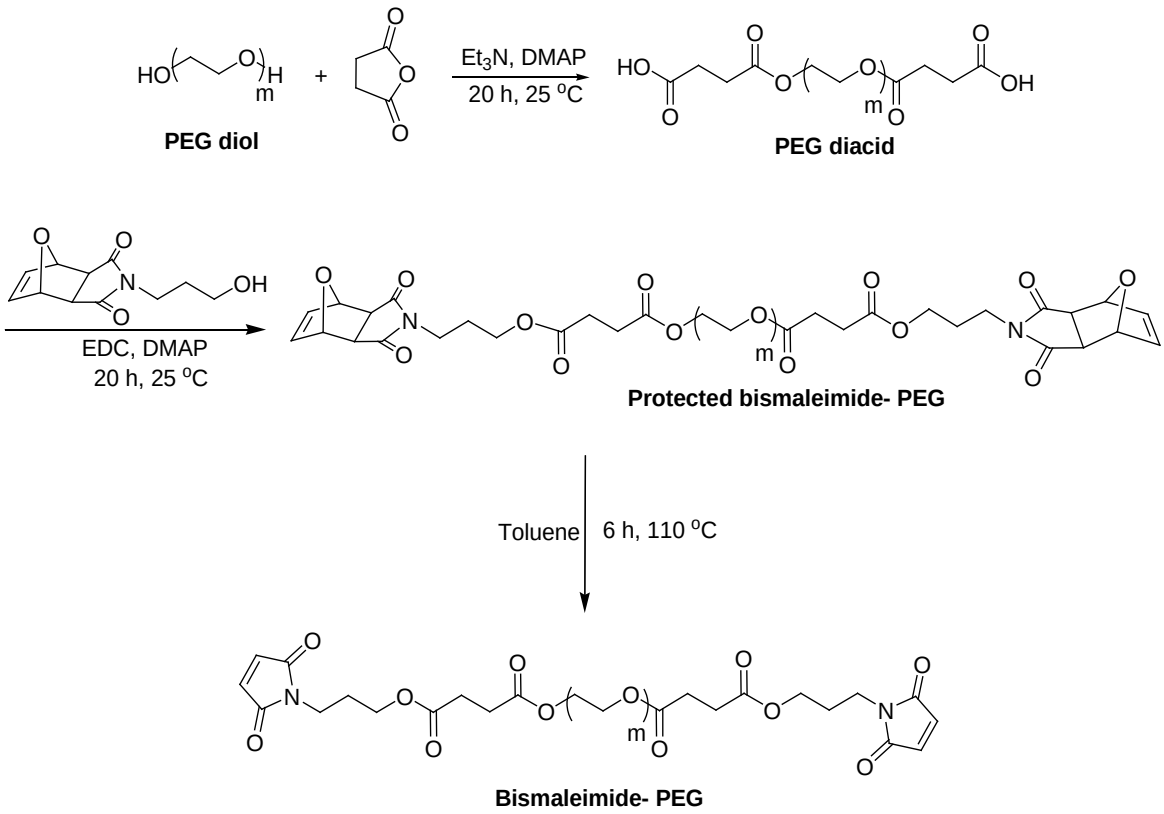


Figure 3.3. Synthesis of maleimide end-functionalized PEGs (PEG-*bm*).

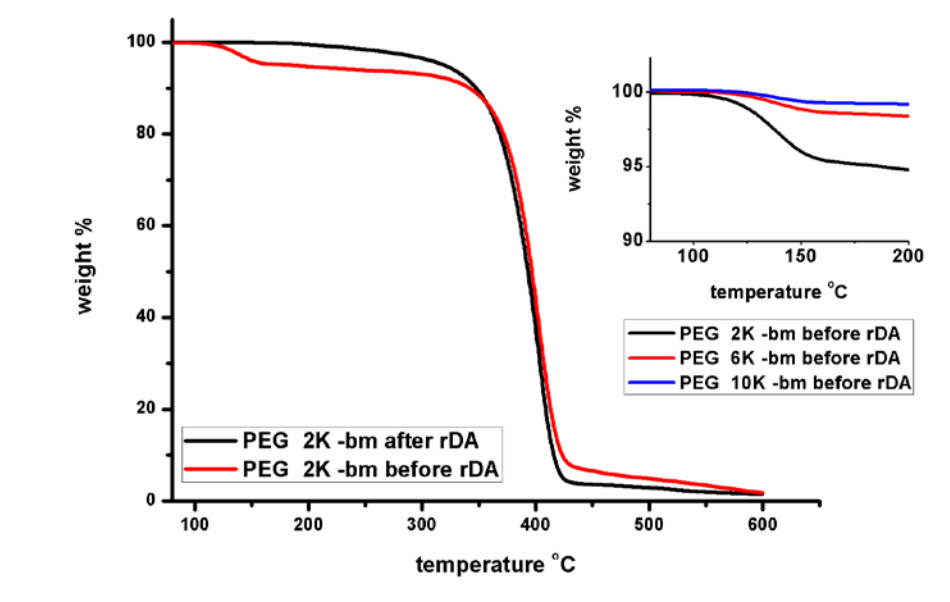


Figure 3.4. TGA thermograms of PEG 2K-*bm* polymer before and after rDA reaction. Upper thermogram shows the comparison of 2K, 6K and 10 K polymers in terms of weight loss.

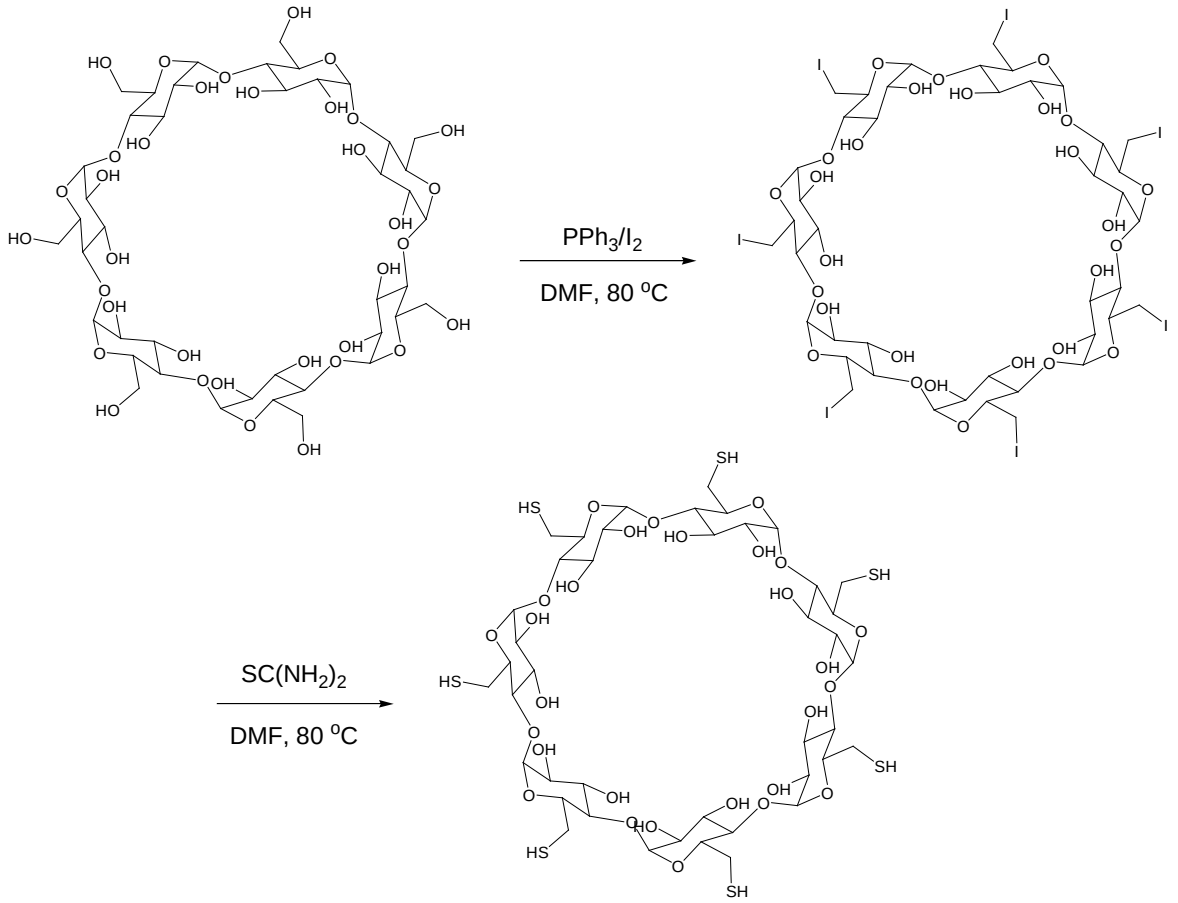


Figure 3.5. Synthesis of thiol-functionalized β-CD (β-CD(SH)$_7$).

3.3.2. Synthesis and Characterization of Hydrogels

Hydrogels were prepared by simply mixing the solutions of PEG-bismaleimide polymers and β-CD(SH)$_7$. Rapid crosslinking occurs through multiple Michael additions between maleimide groups and thiols. Gel formation occurs within a minute in the presence of Et_3N as a catalyst. In order to compare the effect of polymer chain length and crosslinker ratio on physical and morphological properties, a library of hydrogels was prepared (Table 3.1). PEG contents were varied in molecular weight as 2000, 6000 and 10000 gmol^{-1}. The ratio of β-CD(SH)$_7$ crosslinker and PEG-bismaleimide was adjusted to obtain hydrogels with varying crosslinking density and reactive functional group composition.

Table 3.1. Series of hydrogels having different crosslinker ratio and gel conversions of synthesized hydrogels.

Item	Hydrogel	Polymer	Feed Ratio [SH] : [Mal]	Gel Con. (%)	Thiol Content[a] (mmol x10^{-4})	Thiol Cons. (%)
1	$CDP2_{(1:1)}$	PEG 2K-*bm*	1 : 1	89.0	36.10 / 4.62 (±1.07)	87.20
2	$CDP6_{(1:1)}$	PEG 6K-*bm*	1 : 1	87.0	14.70 / 2.26 (±0.83)	84.60
3	$CDP10_{(1:1)}$	PEG 10K-*bm*	1 : 1	86.0	9.28 / 1.73 (±0.67)	81.30
4	$CDP6_{(2:1)}$	PEG 6K-*bm*	2 : 1	92.0	28.07 / 16.92 (±3.39)	39.70[b]
5	$CDP6_{(1.5:1)}$	PEG 6K-*bm*	1.5 : 1	88.0	21.60 / 9.63 (±3.64)	55.40[c]
6	$CDP6_{(1:2)}$	PEG 6K-*bm*	1 : 2	80.0	7.59 / 0.59 (±0.24)	92.20
7	$CDP6_{(1:1.5)}$	PEG 6K-*bm*	1 : 1.5	82.0	10.02 / 1.14 (±0.22)	88.60

[a] Thiol amount used for synthesis of 5 mg hydrogel / Determined thiol amount in 5 mg hydrogel sample (Data in triplicate). [b] Maximum expected value is 50 %. [c] Maximum expected value is 66.67 %.

In the first series of hydrogels, an equimolar stoichiometry of thiol-maleimide was used in the feed. Ideally, upon complete consumption of all reactive functional groups this should provide and hydrogels cannot undergo effective post-polymerization functionalization. To obtain hydrogels that would allow covalent functionalization, a second series of hydrogels was synthesized where the amount of crosslinker was doubled so that all the maleimide groups would combine with thiols and free thiol functionalities will be present in the gel network. A third series of hydrogels were designed to contain free maleimide groups in the network. Such hydrogels were obtained by adjusting the stoichiometry in the feed to contain excess maleimide functional group. The main advantage of this approach is the simplicity for obtaining either thiol or maleimide functionalized hydrogels.

Hydrogels reported here are obtained using well-defined building blocks with specific crosslinking chemistry. A high degree of both physical and chemical homogeneity can be expected for these hydrogels when compared to traditional gels obtained through random crosslinking of polymer chains. The extent of near-ideal network formation can be probed by determination of residual functional groups within the hydrogel. Quantification of residual thiol groups in these hydrogels will provide information about the deviation from ideal network structure expected when equimolar stoichiometry of thiol and maleimide functional groups is employed for gel formation. In order to gain a quantitative

information about the number of sulfhydryl groups, hydrogels prepared using PEGs with varying chain length were treated with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent). The reaction of DTNB with sulfhydryl groups yields a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB) (Figure 3.6). The colored TNB species has a high molar extinction coefficient in the visible range (412 nm); thus sulfhydryl groups can be quantified by reference to the extinction coefficient of TNB.

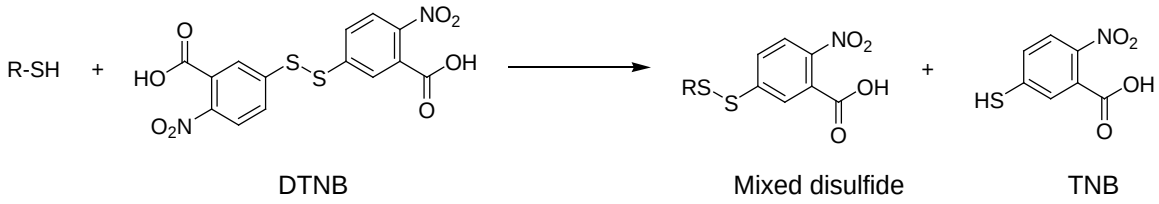


Figure 3.6. Reaction of Ellman's reagent with sulfhydryl groups.

First, samples of hydrogels $CDP2_{(1:1)}$, $CDP6_{(1:1)}$ and $CDP10_{(1:1)}$ were evaluated in terms of free thiol groups in the gel networks. Since stoichiometrically equivalent amount of maleimide and thiol groups were utilized for hydrogel formation, ideally, complete consumption of sulfhydryl groups is expected. However, due to the steric bulk of the polymer chains and steric crowding around the crosslinking sites, some of thiol groups may remain unreacted and the amount of these thiols can provide information about the deviation from ideal network structure (assuming that formation of loops and cycles of polymer chains with crosslinker is low). Equal weights of freshly prepared hydrogel samples were treated with corresponding DTNB in 2.5 mL of PBS buffer and after incubation the absorbance at 412 nm was measured using a spectrophotometer. Calculation of residual thiol content within the hydrogels indicated that 80-90 % of thiol groups were consumed during network formation (Table 3.1). It was noted that increasing the polymer chain length caused a decrease in overall thiol consumption. The hydrogels $CDP6_{(2:1)}$ and $CDP6_{(1:2)}$ with different β-CD crosslinker ratio were also treated with DTNB to gain information about the amount of free thiol groups. A higher thiol consumption was observed in $CDP6_{(1:2)}$ hydrogel than $CDP6_{(1:1)}$. This can be attributed to the presence of higher maleimide feed ratio compared to thiol groups which leads to increased thiol consumption.

Hydrogels were obtained as clear and transparent samples (Figure 3.7f). The microstructures of these hydrogels were examined with ESEM on freeze-dried hydrogel samples. As shown in Figure 3.7, hydrogels were highly porous and possess relatively uniform microstructure suggesting that gelation reactions were homogenous. An increase in apparent pore size was observed upon increase of the PEG chain length that results in decreased crosslinking density. It was also observed that decreasing the crosslinker ratio provides PEG-rich hydrogels as suggested by the fleshy textures along the network.

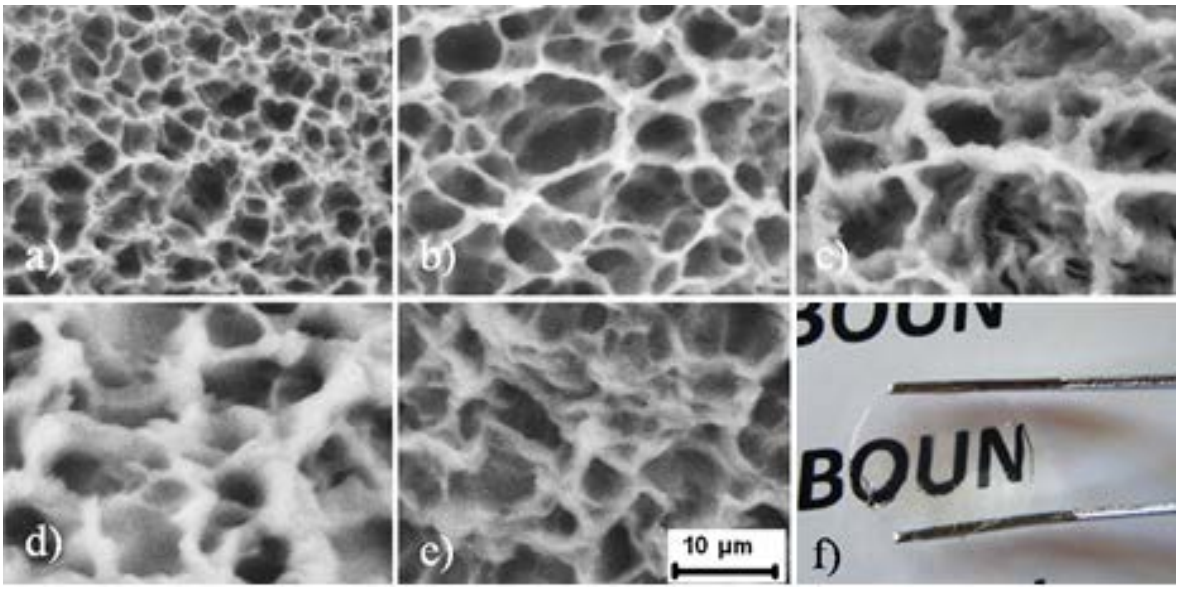


Figure 3.7. Representative ESEM images of freeze-dried hydrogels:
a) $CDP2_{(1:1)}$, b) $CDP6_{(1:1)}$, c) $CDP10_{(1:1)}$, d) $CDP6_{(1:2)}$, e) $CDP6_{(2:1)}$, f) Photograph of transparent hydrogel $CDP6_{(1:1)}$. Scale bar for all images is 10 µm.

Swelling properties of the hydrogels were probed gravimetrically by recording water uptake over time until they reached equilibrium. Firstly, water uptake of hydrogels prepared by PEGs of increasing molecular weights (ca. 2000, 6000 and 10000 $gmol^{-1}$) was investigated. Swelling profiles show that hydrogels reach equilibrium swelling rapidly and the highest swelling ratio was dependent on the chain length of the PEG polymer (Figure 3.8a). As expected, decreased crosslinking density and increased hydrophilicity due to the increased chain length results in higher water uptake. Comparative water uptakes of $CDP6_{(1:1)}$, $CDP6_{(2:1)}$ and $CDP6_{(1:2)}$ hydrogels with different crosslinking degrees demonstrate that the highest swelling ratio belongs to the hydrogel $CDP6_{(1:2)}$ which has lowest degree of crosslinking as well as the highest PEG content (Figure 3.8b).

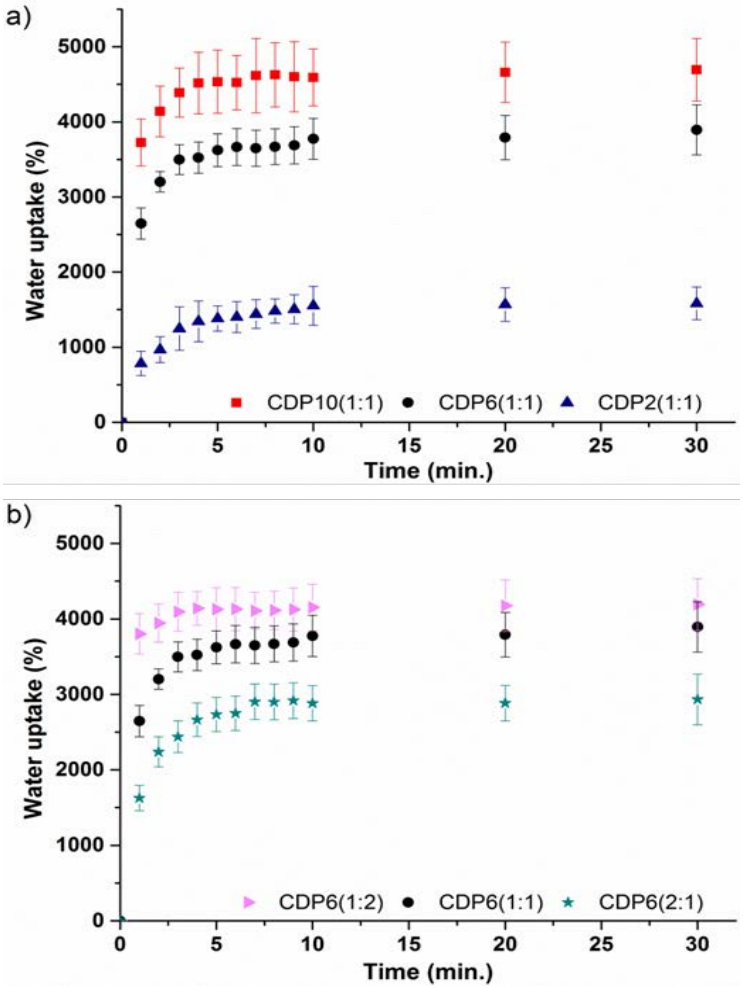


Figure 3.8. Water uptake of hydrogels in water versus time for comparison of hydrogels as a function of a) PEG chain length, b) crosslinker ratio. All data are in triplicate.

Hydrogels were evaluated in terms of their rheological properties via dynamic frequency scan measurements of water-swollen samples. It was found that the value of storage modulus (G′) that reflecting the elastic properties and loss modulus (G″) reflecting viscous behavior ranged from 10 to 10^3 Pa (Figure 3.9). In all cases, G′ values are higher than G″ values which is characteristic for hydrogels. The angular frequency change had relatively low effect on the storage and loss moduli indicating the elastic solid behavior. A direct relationship between the polymer molecular weight and G′ values was observed. More enhanced viscoelastic properties were obtained in case of using lower molecular weight polymer. The viscoelastic properties of hydrogels were also modulated by changing the feed of β-CD crosslinker, whereby an increase in the β-CD crosslinker content resulted in increased storage moduli.

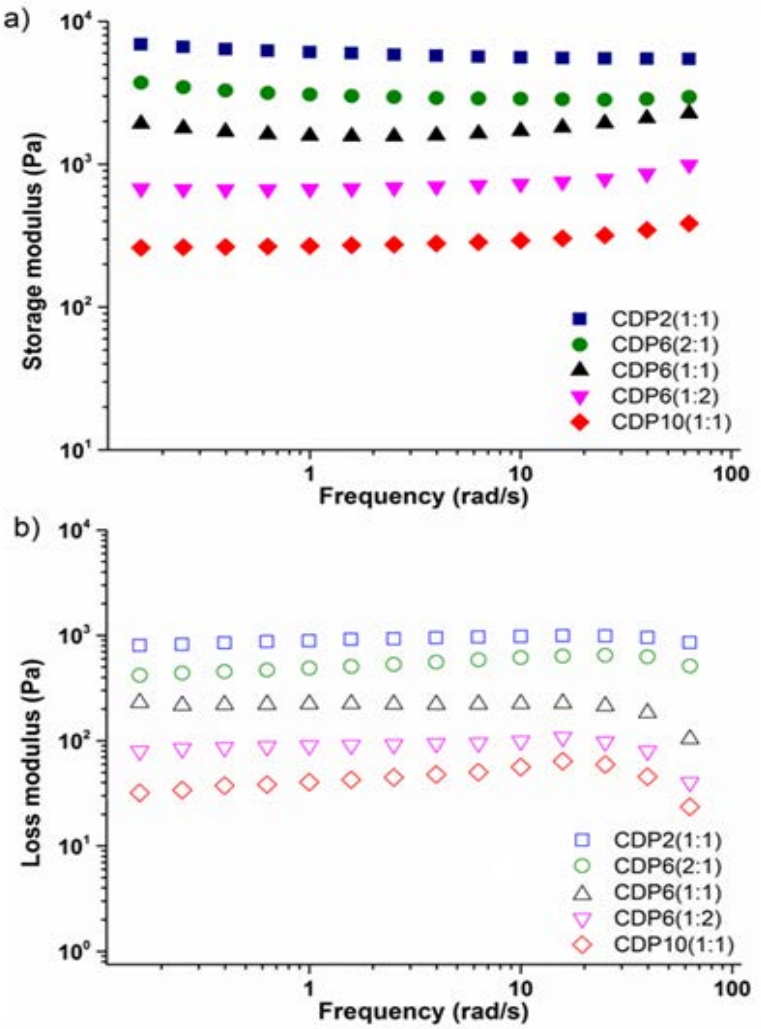


Figure 3.9. The frequency dependence of a) storage moduli and b) loss moduli for the hydrogels.

3.3.3. Functionalization of Hydrogels

Since hydrogels are important scaffolds for biomolecule immobilization and drug delivery, it is important to have reactive units in gel interior to attach desired agents. Thus, post-gelation functionalization efficiency of synthesized hydrogels was examined by attaching fluorescent dyes onto the reactive groups in the gel matrix. The main advantage of this approach is the simplicity for obtaining either thiol or maleimide functionalized hydrogels, since by changing β-CD(SH)$_7$ ratio, desired amount of free functional groups can be placed in the network.

In the first series of hydrogels crosslinker ratio was adjusted to have free thiol groups and the functionalization of the thiol groups were studied by conjugation of maleimide

functionalized fluorescein dye. The series of hydrogels $CDP6_{(1:1),}$ $CDP6_{(1,5:1)}$ and $CDP6_{(2:1)}$ with increasing crosslinker ratio (increasing free thiol content) were selected and reacted with excess fluorescein maleimide. After removing excess dye with successive washings, extent of immobilization was investigated using fluorescence microscopy. As expected, hydrogels having higher amounts of free thiol groups were able to immobilize higher amounts of fluorescein maleimide dye (Figure 3.10a). Control experiment which was done by incubation of hydrogel $CDP6_{(2:1)}$ with fluorescein amine resulted lack of any significant fluorescence. These results show that free thiol groups in the hydrogel allow the covalent attachment of model dye molecules.

To demonstrate the tunability of functionalization of the hydrogel using thiol containing molecules a series of $CDP6_{(1:2)}$, $CDP6_{(1:1,5)}$ and $CDP6_{(1:1)}$ hydrogels were selected and reacted with thiol containing fluorescent dye. After incubation, hydrogels were washed to remove excess dye and fluorescent microscope images were taken. A direct correlation was observed between free maleimide groups in the hydrogels and fluorescence intensity (Figure 3.10b). As a control experiment, the hydrogel $CDP6_{(1:2)}$ was incubated with fluorescein maleimide instead of fluorescein thiol. Analysis with fluorescence microscopy revealed that there was minimal attachment of dye, thus indicating the high consumption of thiol groups, as intended during fabrication.

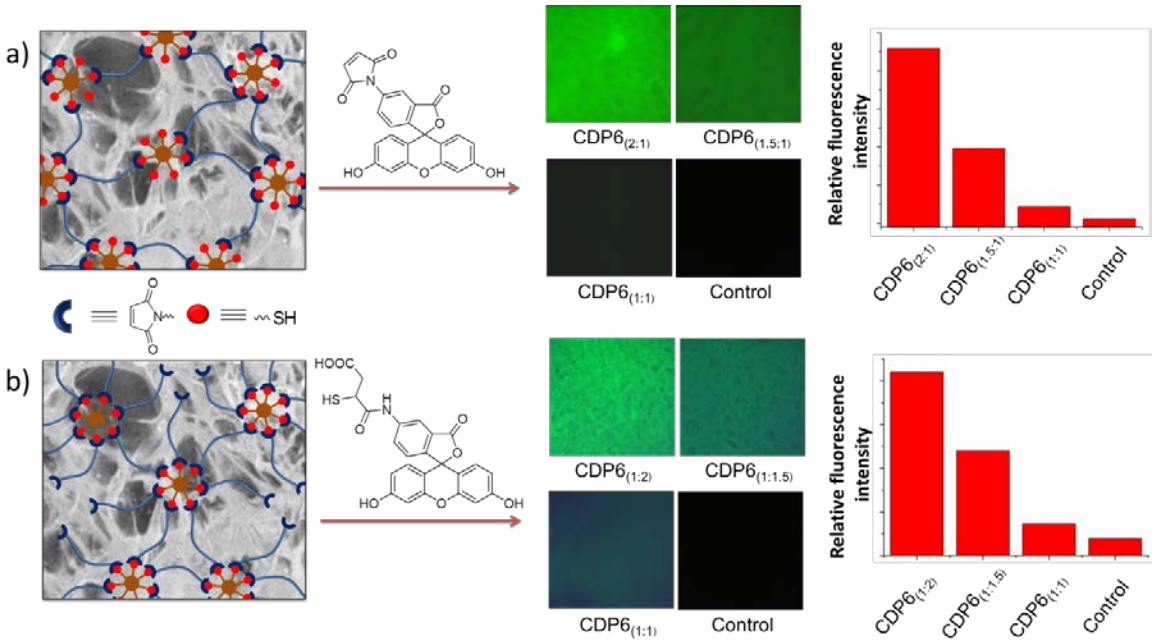


Figure 3.10. Functionalization of hydrogels with fluorescent dye molecules: a) Functionalization of thiol-containing hydrogels with maleimide-containing fluorescein dye b) Functionalization of maleimide-containing hydrogels with thiol-containing fluorescein dye.

3.4. Conclusions

In this study functionalizable hydrogels with well-defined network structures were prepared using the thiol-maleimide conjugation reaction between telechelic maleimide functionalized linear PEGs and thiol functionalized β-cyclodextrin. Clear and transparent hydrogels are rapidly formed with high gel conversions. Water uptake properties of these hydrogels were found to be dependent on the molecular weight of PEG and the crosslinker ratio. The amount of CD-based crosslinker was varied to obtain hydrogels containing reactive maleimide or thiol functional groups within the hydrogel matrix. Maleimide containing hydrogels were efficiently functionalized with thiol-containing fluorescein dye and extent of immobilization onto the hydrogels was dependent on the amount of free maleimide groups in gel matrix. Likewise, hydrogels containing free thiol groups were functionalized with maleimide containing dye molecules with high efficiency. One can expect that functionalizable hydrogels thus obtained from easily accessible precursors using effective gelation under benign conditions will find usage in various areas of biomedical sciences. The hydrogel synthesis methodology depicted here allows

incorporation of CD units that would allow host-guest interactions with hydrophobic molecules and is believed to find potential application in design of controlled drug release systems.

4. FABRICATION OF POLY(ETHYLENE GLYCOL)-BASED CYCLODEXTRIN-CONTAINING HYDROGELS VIA THIOL-ENE CLICK REACTION

The materials in this chapter have been adapted with modifications from the following article: Arslan M., T. N. Gevrek, R. Sanyal, A. Sanyal, *European Polymer Journal*, Vol. 62, pp. 426-434, 2015.

4.1. Introduction

As discussed in previous sections, hydrogels have been gaining increased relevance in biomedical applications including the design of controlled and sustained drug release formulations, building up of the scaffolds for tissue regeneration and substitution, solid-phase biocatalysts, design of biomolecular sensors, implant materials and implant coatings. The porous hydrophilic network structures of hydrogels allow the absorption of large amount of water while retaining their viscoelastic behavior. This ability to swell, high biocompatibility and tunable mechanical properties make hydrogels suitable materials for use as controlled drug delivery platforms.

The voids in hydrogel interior serve as reservoirs to load the drugs and release them in a sustained manner through different mechanisms such as diffusion, erosion, matrix relaxation and degradation [114]. Different approaches have been developed to increase the loading efficiency of hydrogels through covalent, hydrophobic, ionic interactions with drug molecules, as well by employing molecular imprinting techniques [115]. Hydrogels are also suitable platforms in immobilization of biomolecules and development of immunoassays. Due to the high surface area and ability of tailoring network structure for functionalization with receptor ligands, hydrogels possess important utility in biomolecular immobilization. Development of efficient methodologies for controlling the hydrogel network structure or covalent or non-covalent immobilization of bioactive compounds for aforementioned applications is an important area in soft material design.

The fabrication of hydrogels can be accomplished using several methods, including physical or chemical crosslinking of hydrophilic polymers. Chemical crosslinking of hydrogels is based on the formation of covalent bonds between polymers or monomers and offer more stable network formation than the physically crosslinking. Chemically cross-linked hydrogels are synthesized using various chemical reactions and techniques, the techniques involve the polymerization of monomers in the presence of a crosslinking agent. In recent years, by the advancements of new synthetic methodologies, a vast range of chemical reactions have been adapted towards hydrogel synthesis. Especially, click chemistry based methodologies for covalent crosslinking has been utilized to prepare hydrogels for wide range of applications [116]. Various click strategies including copper catalyzed click chemistry, Diels-Alder reaction, and thiol-ene reactions were successfully employed in crosslinking of monomers and polymers carrying complementary functional groups. Due to the highly effective and selective nature of click reactions, hydrogels prepared in this way possess more controlled and complete networks with improved physical properties.

Among the various available click reactions, the metal-free thiol-based conjugation strategies are attractive for fabrication of materials intended for biological applications. Thiols can undergo various click reactions that precede under mild conditions with near quantitative conjugation efficiencies. The reaction of a thiol with an alkene group under radical-initiated conditions is known as radical thiol-ene reaction. The reaction is based on the formation of thiyl radicals; which are generated in situ from the corresponding thiols using a radical source. Due to high selectivity, high reaction conversions and mild reaction conditions this chemistry has been proven as a versatile methodology for preparation of cross-linked networks [16]. Using radical thiol-ene reaction, network formation is controlled via spatial and temporal regulation of polymerization allowing the homogeneous structures [117]. The reaction can also proceed in presence of air or in aqueous environment thus providing a powerful technique for fabrication of hydrogels for biomedical applications.

Cyclodextrins (CDs), a family of cyclic oligosaccharides consisting of 1,4-linked D-glucopyranose units are torus shaped molecules characterized by a hydrophilic outer shell and a hydrophobic cavity interior. The unique structure of CDs allows the incorporation of

hydrophobic molecules into their inner cavity through inclusion complex formation (Figure 4.1). The interaction of cyclodextrins with hydrophobic molecules is a dynamic equilibrium, rendering the formation and dissociation of inclusion complex dependent on environmental conditions [63]. In the presence of a competitive host molecule or dilution of the aqueous environment, the molecule included in the cavity can be released.

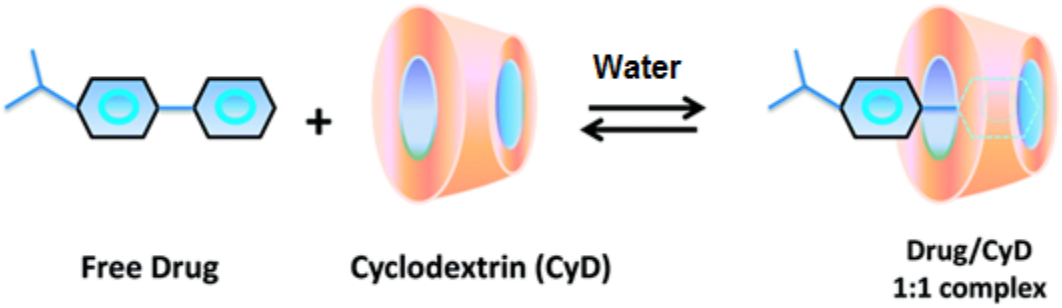


Figure 4.1. General representation of inclusion complex formation between a cyclodextrin and a hydrophobic molecule (From [118] with permission).

Due to the ability of reversible complex formation with hydrophobic molecules, cyclodextrins are extensively studied in the design of polymer/hydrogel based sustained drug delivery systems to modify the release kinetics [119]. Common strategies for incorporating cyclodextrin units into hydrogels network include the polymerization of cyclodextrin containing monomers with other hydrophilic monomers or by reacting hydroxyl groups with isocyanate or epoxide containing polymers, as depicted in the introduction part. In the latter approach the multivalent chemical structure of cyclodextrins allows them to be used as multifunctional crosslinking reagents. However in these systems the crosslinking occurs through participation of hydroxyl groups from both the top and bottom rim hydroxyl groups of the cyclodextrin in a random fashion that leads to heterogeneity. Thus, another strategy for cyclodextrin incorporation to hydrogel networks includes the use of chemically modified cyclodextrin from primary hydroxyl groups. This strategy allows the utilization of various chemical reactions for gel synthesis by choosing proper functional groups on cyclodextrin and hydrophilic polymers.

Microstructured hydrogels are actively investigated since it is well documented that relief size and shape affects how these materials interact with their environment. For

example, it is known that micropatterns on hydrogel surfaces modulate cellular proliferation and adhesion [120]. Hydrogel based micro-needles allow sustained drug delivery over prolonged period to time [120, 121]. Due to its operational simplicity, efficiency and high selectivity, photo-chemically governed radical thiol-ene click reaction is an ideal method for obtaining micropatterned hydrogels.

In the present study, synthesis of poly(ethylene glycol) (PEG)-based cyclodextrin containing hydrogels through radical thiol-ene reaction is described [123]. The strategy is the utilization of alkene end-functionalized poly(ethylene glycol)s as the hydrophilic matrix and thiol functionalized β-cyclodextrin (β-CD(SH)$_7$) as multifunctional crosslinker. Structurally well-defined hydrogels were obtained in high conversions through rapid gelation reactions under mild conditions. In order to tailor the physicochemical properties of hydrogels, polymer molecular weight and crosslinking degrees were varied. The resulting hydrogels were characterized by their water uptake properties and morphology, as well as their dynamic rheological behavior. These cyclodextrin embedded hydrogels were investigated for uptake and release of a poorly water-soluble drug puerarin used in the treatment of glaucoma. Additionally, microstructures were fabricated to highlight the advantage of the photochemically induced gelation process.

4.2. Experimental

4.2.1. Materials and Characterization

Poly(ethylene glycol)s (PEG 2 K, 4 K, 8 K), allyl bromide, 2,2-Dimethoxy-2-phenylacetophenone (DMPA), (5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) and (Trimethylsilyl)methacrylate (TMSMA) were obtained from Aldrich Chemical Co. Puerarin was obtained from TCI Chemicals. Other chemicals and solvents were purchased from Merck and used as obtained without further purification unless otherwise noted. Synthesis of thiol functionalized β-cyclodextrin [109] and α,ω-diallyl PEGs [124] were conducted according to reported procedures. All thiol-ene reactions were performed at 365 nm using a handheld UV lamp (Blak-Ray UVP model B-100AP/R high intensity UV lamp with a 100-watt spot bulb and 7° beam width).

4.2.2. Representative Hydrogel Formation via Radical Thiol-Ene Reaction

Hydrogels syntheses were accomplished by multiple thiol-ene reactions between thiol-functionalized β-cyclodextrin (β-CD$(SH)_7$) and diallyl-PEGs. The polymer (0.050 g, 12.2 x 10^{-3} mmol for *dially*-PEG_{4000}) was placed in a vial and dissolved in DMF (100 µL). A calculated amount of β-CD-$(SH)_7$ and photoinitiator, DMPA (0.2 eq. per thiols) were then added to this solution. The mixture was UV irradiated for 30 minutes at 365 nm. After hydrogel formation, unreacted species were removed by washing the gel with DMF followed by distilled water several times. The swollen hydrogel sample was frozen and freeze-dried in vacuo to yield dried hydrogel.

4.2.3. Determination of Thiol Content

Free thiol group contents of obtained hydrogels were determined using Ellman's method [112]. The sulfhydryl groups were quantitated by reference to the extinction coefficient of TNB^{2-} (2-nitro-5-thiobenzoic acid) ion. A reaction medium of 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA buffer was prepared. Ellman's reagent solution was prepared by dissolving 4 mg Ellman's reagent (5,5'-Dithio-*bis*-(2-nitrobenzoic acid) in 1 mL of reaction buffer. 5 mg sample of hydrogel was placed in a vial and 2.5 mL of buffer and corresponding Ellman's reagent solution were added onto the solution. The resultant mixture was incubated at 37 °C for 2 hours. The absorbance at 412 nm was measured to calculate the total sulfhydryl group content in the sample using the molar extinction coefficient of TNB^{2-} (14,150 $M^{-1}cm^{-1}$).

4.2.4. Swelling Studies

Swelling studies were conducted by sampling certain amount of hydrogel in a vial containing distilled/deionized water at room temperature. The increase in mass of sample was followed as a function of time till the hydrogels showed constant weight. The percentage weight change was obtained from fractional weight change using the empirical relationship:

$$\text{Percentage of \% Swelling} = (W_{wet} - W_{dry}) / W_{dry} \times 100,$$

where W_{wet} and W_{dry} refer to the weight of wet and dry hydrogels respectively. The measurements were made in triplicate and average data was used for plotting swelling curves.

4.2.5. Scanning Electron Microscopy (SEM)

Hydrogels were analyzed with scanning electron microscopy (SEM) studies in order to characterize their morphologies. SEM images of dried hydrogels were taken using an ESEM-FEG/EDAX Philips XL-30 (Philips, Eindhoven, The Netherlands) instrument using an accelerating voltage of 10 kV.

4.2.6. Rheological Measurements

Rheological behaviors of hydrogels were evaluated in triplicate by measuring the loss (G″) and storage (G′) moduli of the swollen hydrogel as a function of angular frequency using an Anton Paar MCR 302 rheometer. Tests were carried out at 25 ^{o}C by applying 0.5 % strain between 0.1-100 rad/s. The hydrogel samples prepared as disk were analyzed with a parallel plate of 8 mm diameter and the gap between plates was adjusted to 2.0 mm.

4.2.7. Drug Loading and Release Studies

Puerarin was loaded into the hydrogels using a solution absorption method. Hydrogel disks (~0.1 g) were soaked in 50 mL 0.80 mg/mL puerarin solution at 37 ^{o}C and the solution was gently vibrated at 100 rpm. The drug loading was monitored by analyzing the puerarin concentration in the soaking solution using a UV–vis spectrophotometer at 250 nm.

The puerarin loaded hydrogels were rinsed with distilled water and added to 15 mL distilled water as the release medium. The release studies were carried out at 37 ^{o}C with a 100 rpm mechanical shaking. With predetermined time intervals, 5 mL of release medium was taken out and replaced with same volume of fresh water. The concentration of drug in collected media was monitored spectrophotometrically. The loading capacities of hydrogels were expressed in terms of cumulative release.

4.2.8. Fabrication of Patterned Hydrogels

Silicon surfaces were cleaned by sonicating in detergent, deionized water, acetone and isopropyl alcohol and were dried in an oven. Thereafter they placed in ozone cleaner for 30 minutes. Freshly cleaned Si/SiO_2 surfaces were dipped in 1 % solution of TMSMA in anhydrous toluene under a nitrogen atmosphere for 12 h. After washing several times

with toluene and then with methanol, surfaces were dried under a stream of nitrogen. 10 µL of $P4CD_{(2:1)}$ hydrogel precursor solution prepared with 30 mg (7.35 $x10^{3-}$ mmol) diallyl-PEG_{4000}, 5.23 mg (4.2 $x10^{-3}$ mmol) β-$CD(SH)_7$, 1.5 mg (5.38 $x10^{-3}$ mmol, 0.2 eq per thiol) DMPA and 500 µL DMF was spin-coated on a 1x1 cm^2 modified Si/SiO_2 wafer at 500 rpm for 18 seconds and subsequently for 180 seconds at 1000 rpm. A TEM grid photomask was placed on the precursor surface and UV light at 365 nm was exposed from a distance of 10 cm for 30 minutes. After removing the TEM grid, to remove any unreacted species, surface was washed several times with THF.

4.3. Results and Discussion

4.3.1. Synthesis and Characterization of Hydrogels

Poly(ethylene glycol)-based hydrogels were obtained via photo-initiated radical thiol-ene reaction of diallyl-functionalized PEG polymers (*diallyl*-PEGs) with a heptavalent thiol-functionalized β-cyclodextrin crosslinker (β-$CD(SH)_7$) (Figure 4.2). The network formation reactions were carried out under 365 nm UV irradiation in the presence of photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DMPA). After exposure of UV light, soft and transparent hydrogels were obtained in good yields. In order to find out the effect of polymer chain length and amount of crosslinker on hydrogel characteristics, a series of hydrogels were prepared. The hydrogel compositions, feed ratios and gel conversion values are tabulated in Table 4.1.

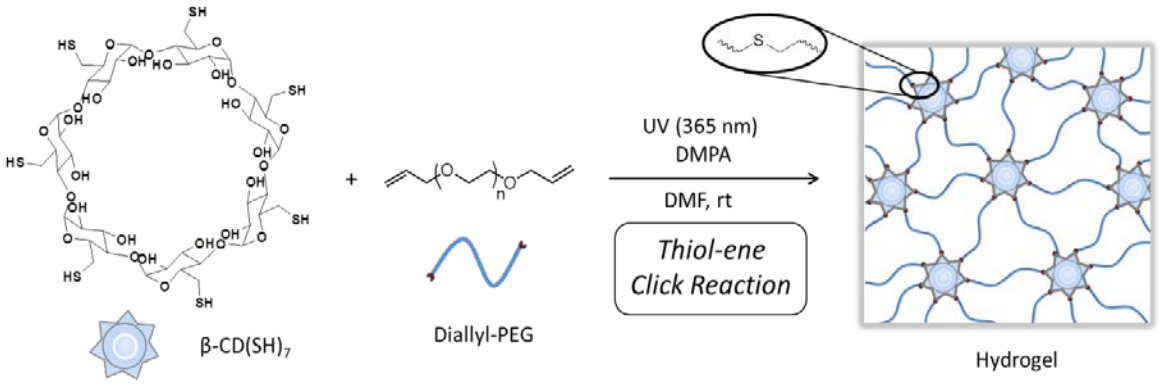


Figure 4.2. Schematic illustration of hydrogel synthesis via radical thiol-ene click reaction.

Table 4.2. Properties of hydrogels with varying polymer molecular weights and crosslinker ratio.

Hydrogel	Polymer	Feed Ratio [-SH]:[alk.]	Conv. (%)	Thiol Content[a] (mmol x10^{4-})	Thiol Cons. %	Drug Loading[b] (mg/g dry gel)
$P2CD_{(1:1)}$	*diallyl*-PEG_{2000}	1 : 1	92.0	41.0 / 5.61 (±0.77)	86.3	34.72 ± 1.41
$P4CD_{(1:1)}$	*diallyl*-PEG_{4000}	1 : 1	87.0	22.0 / 3.80 (±0.46)	82.7	26.32 ± 1.57
$P8CD_{(1:1)}$	*diallyl*-PEG_{8000}	1 : 1	84.0	11.5 / 2.58 (±0.58)	77.5	30.83 ± 2.95
$P4CD_{(2:1)}$	*diallyl*-PEG_{4000}	2 : 1	93.0	41.0 / 23.41 (±2.38)	42.9	32.29 ± 2.23
$P4CD_{(1:2)}$	*diallyl*-PEG_{4000}	1 : 2	79.0	12.0 / 1.03 (±0.21)	91.4	28.96 ± 2.59

[a] Thiol amount used for synthesis of 5 mg hydrogel / Determined thiol amount in 5 mg hydrogel sample (Data in triplicate). [b] Drug loading of hydrogels in 0.80 mg/mL soaking solution of puerarin (Data in triplicate).

To understand the polymer molecular weight of the polymeric segment on hydrogel properties, dially-functionalized PEGs with varying molecular weight as ~2K, 4K and 8K were used. In each case, the feed ratio of polymer and crosslinker was adjusted to one to one coupling of alkene groups with thiols (Table 4.1, Entry 1,2,3). Ideally, complete pairing of alkene groups with thiols would allow the formation of well-defined networks. However, due to the steric bulk of polymer chains, cyclizations, side reactions and steric crowding around the crosslinking sites, deviance from the ideal network formation would be expected. In case of using different molecular weight polymers, a relatively higher conversion was obtained with lower chain length polymer. Increasing polymer length caused lower gel conversions. In order to understand the efficiency of the crosslinking process, an analysis of residual thiol-group content was undertaken. The consumption of thiol functional groups during network formation was accounted via performing colorimetric sulfhydryl assay using 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), Ellman's reagent. The thiol amount used in hydrogel formation, determined ratio and percent thiol consumption of hydrogels are reported in Table 4.1. The hydrogels were freshly prepared and analyzed to minimize the disulfide formation of thiol groups. According to the results,

an increasing trend in percent thiol consumption was observed with lowering the polymer molecular weight.

A series of hydrogels with chancing crosslinker ratio were prepared from *dially*-PEG_{4000} polymer. In addition to (1:1) feed of thiol and alkene functionalities, the stoichiometric ratio between these groups were tailored as (2:1) and (1:2) to obtain more cross-linked and less cross-linked hydrogel networks, respectively (Table 4.1, Entry 2,4,5). By comparing conversion values, it was observed that, higher conversions were obtained in case of using higher crosslinker ratio. Although the thiol analysis showed over 90 % thiol consumption in $P4CD_{(1:2)}$ hydrogel, the lowest gel conversion value was attained. This result was attributed the imbalance between number of thiol and alkene groups during gel formation inducing the participation of polymer chains into network.

The swelling behaviors of hydrogels were studied in water by recording the water uptake in pre-determined time intervals until equilibrium swelling was attained. As shown in Figure 4.3, all hydrogels exhibited good swelling properties and fast kinetics. As expected, the swelling ratios of hydrogels have shown high dependence on hydrophilic PEG polymer content. With increasing polymer molecular weight increased swelling ratios were obtained (Figure 4.3a). The effect of crosslinker ratio on swelling degrees was studied with $P4CD_{(1:1)}$, $P4CD_{(2:1)}$ and $P4CD_{(1:2)}$ hydrogels. The highest swelling ratio was obtained for $P4CD_{(1:2)}$ hydrogel which has the lowest degree of crosslinking as well as the highest hydrophilic polymer content (Figure 4.3b).

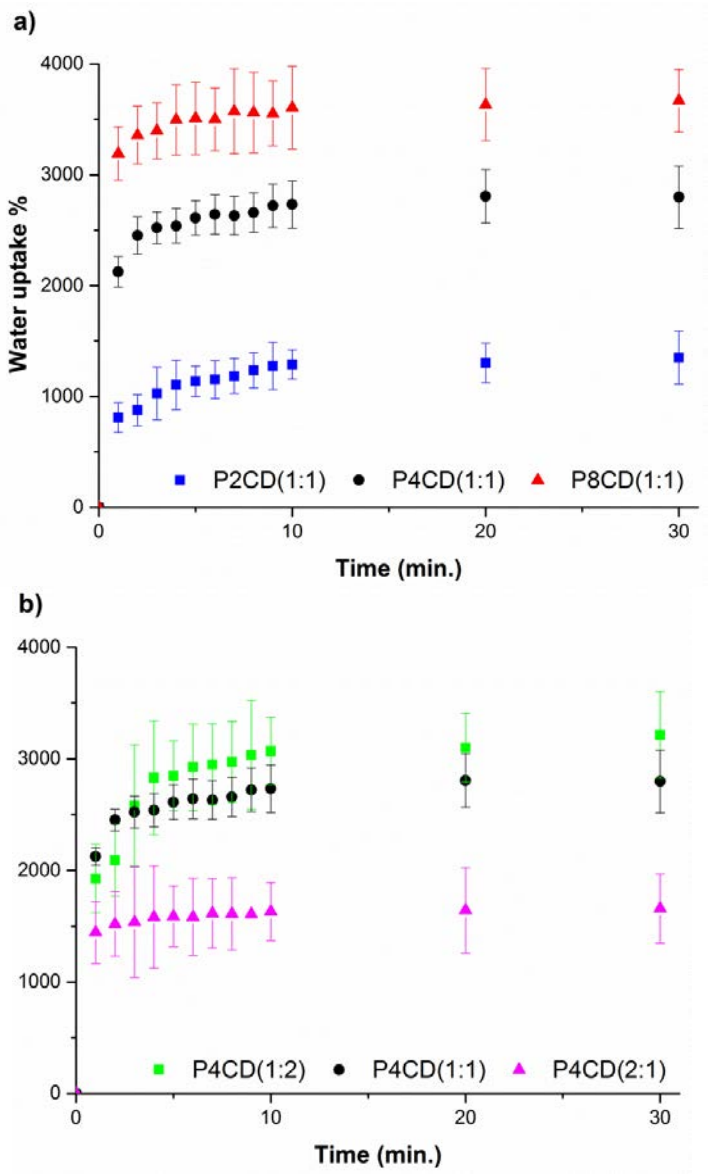


Figure 4.3. Equilibrium swelling ratios of hydrogels in water. Comparison of hydrogels as a function of a) PEG chain length b) crosslinker ratio.

The surface morphologies of obtained hydrogels were investigated using scanning electron microscopy (SEM). The water swollen hydrogels were frozen and vacuum dried before the analysis. As shown in Figure 4.4 interconnected porous structures with varying pore sizes observed in the microphotographs. With increasing chain length of PEG from 2K to 8K and with increasing ratio of crosslinker from 2:1 to 1:2, larger porous structured hydrogels were obtained. These results are coherent to hydrogel swelling characteristics in which hydrogels with larger porous structures show higher water uptake capacity.

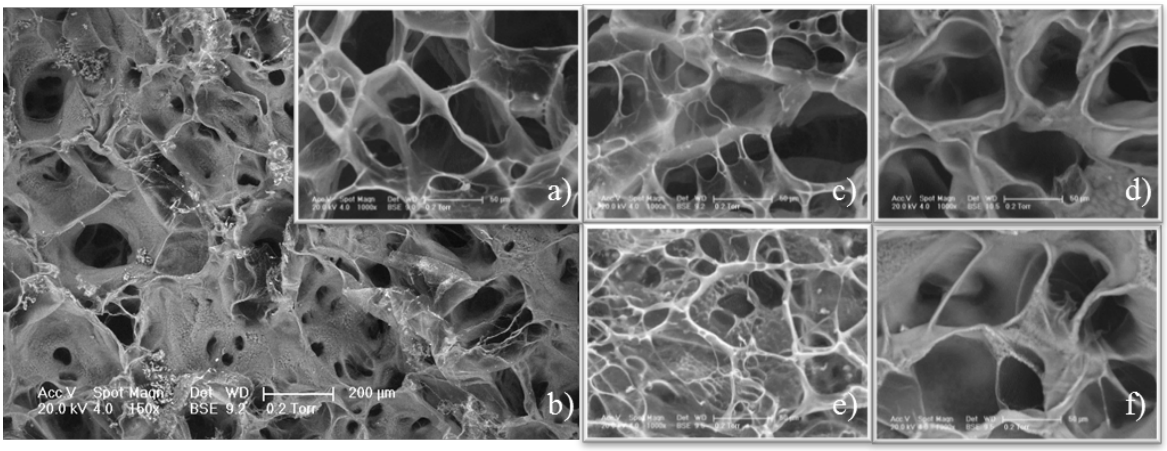


Figure 4.4. Representative ESEM images of freeze-dried hydrogels: a, b) $P4CD_{(1:1)}$, c) $P2CD_{(1:1)}$, d) $P8CD_{(1:1)}$, e) $P4CD_{(2:1)}$, f) $P4CD_{(1:2)}$. Large image scale bar: 200 μm; small images: 50 μm.

The rheological properties of water-swollen hydrogels were investigated via dynamic frequency scan measurements. It was found that hydrogels exhibit storage modulus (G′) and loss modulus (G″) values ranging from 10^0 to 10^3 Pa (Figure 4.5). The storage and loss moduli show low angular frequency dependency indicating the well-structured network formation [65]. Increasing the polymer molecular weight cause a decrease in G′ value and more enhanced viscoelastic properties were obtained with $P2CD_{(1:1)}$ hydrogel containing lowest molecular weight polymer. It has also observed that the feed of β-CD crosslinker in hydrogels composition also affects the rheological properties. Incorporation of higher amount of β-CD crosslinker resulted in stronger viscoelasticity.

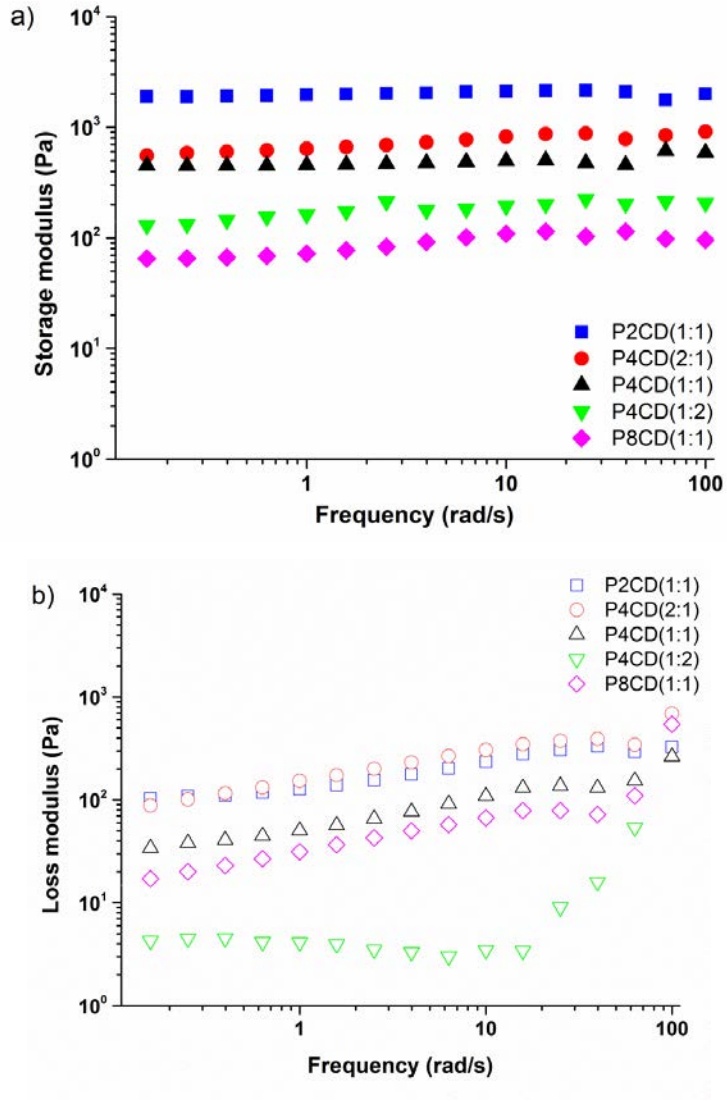


Figure 4.5. a) The frequency dependence of storage moduli for the hydrogels, b) The frequency dependence of loss moduli for the hydrogels.

4.3.2. Drug Loading and Release Studies

The drug loading and release characteristics of hydrogels were examined using poorly water-soluble (0.011 M at 25 ^{o}C, [125]) model drug puerarin (Figure 4.6). The drug was loaded to hydrogel samples prepared as disks using solution absorption method. Pre-water swollen hydrogel disks were immersed in 0.80 mg/mL puerarin solution and the drug loading was monitored by UV-spectrophotometry until equilibrium was reached after 24 h incubation. The total drug amounts absorbed by the hydrogels, determined from the initial and final concentrations of soaking solutions were shown in Table 4.1. The loaded drug amounts were found to be affected by the length of hydrophilic PEG polymer and β-

cyclodextrin crosslinker ratio. Highest drug loading was achieved with $P2CD_{(1:1)}$ hydrogel, containing the smallest PEG polymer at the same time the highest β-cyclodextrin ratio. With increased PEG length in hydrogel $P4CD_{(1:1)}$, lower drug loading was achieved. In this series, $P8CD_{(1:1)}$ hydrogel with highest PEG length and lowest β-cyclodextrin content showed moderate loading capacity. The results could be explained by taking the different loading mechanisms of hydrogels into account. In hydrogels, drugs are mainly diffused in aqueous phase or adsorbed to the polymeric backbones [126]. In cyclodextrin containing hydrogels, the ability of inclusion complexation between cyclodextrin and hydrophobic molecules provide another mean of drug loading. For equal amount of polymer used in hydrogel synthesis, highest amount of β-CD was incorporated in $P2CD_{(1:1)}$ hydrogel. Since puerarin is able to form inclusion complexes with β-cyclodextrin [127], increased β-cyclodextrin ratio in $P2CD_{(1:1)}$ makes a notable contribution to drug loading. Besides, the amount of drug diffused in aqueous phase and adsorbed on hydrogel backbone is affected from the polymer chain length. Increased polymer length causes larger voids in the gel network, thus allowing the higher drug loading. Thus, in $P8CD_{(1:1)}$ hydrogel, increased drug loading compared to $P4CD_{(1:1)}$ hydrogel could be attributed to predominant effect of higher molecular weight polymer. A similar drug loading trend is also observed in hydrogel series $P4CD_{(1:1)}$, $P4CD_{(2:1)}$ and $P4CD_{(1:2)}$ prepared from same polymer but with changing β-cyclodextrin cross-linker ratios. $P4CD_{(2:1)}$ hydrogel having highest content showed highest β-cyclodextrin drug loading.

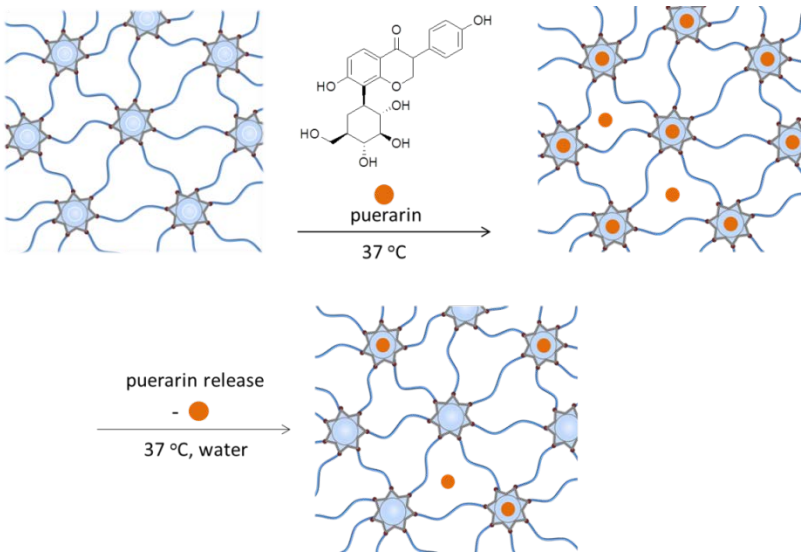


Figure 4.6. Schematic illustration of drug loading to cyclodextrin-containing hydrogels and drug release.

The drug loaded hydrogels were gently washed with distilled water before adding to the release medium. With regular time intervals 5 mL release medium was replaced with a fresh solution and collected release solution was analyzed via UV spectrophotometer to monitor drug release. The release behavior of puerarin from hydrogels is shown in Figure 4.7.

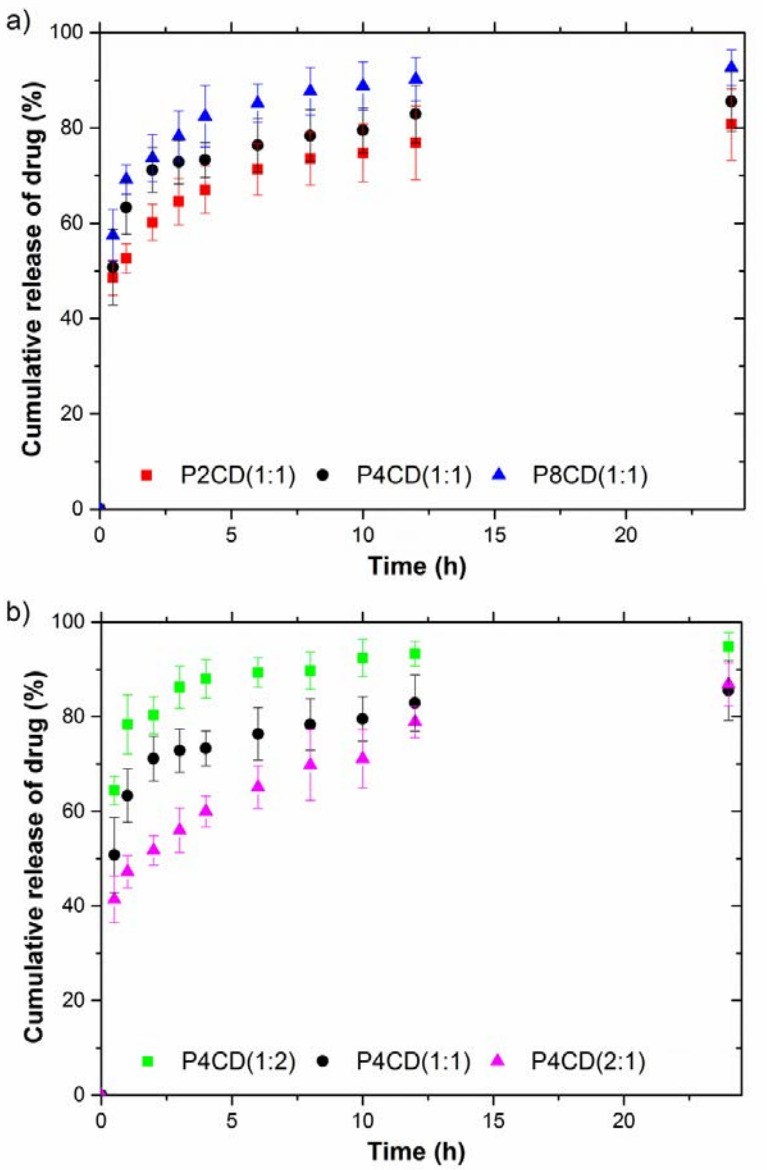


Figure 4.71. Cumulative release of puerarin from hydrogels a) Effect of PEG length b) β-CD crosslinker ratio (± SD, n = 3).

Initially, a burst release of the drug was observed for all hydrogels. This accelerated release is common in hydrogel based release systems and mainly attributed to the fast removal of free drug in aqueous phase and adsorbed drug on backbone of the hydrogel [128]. The amount of burst released drug is increasing with the increase in polymer

molecular weight among hydrogels $P2CD_{(1:1)}$, $P4CD_{(1:1)}$, $P8CD_{(1:1)}$. The β-CD content in these hydrogels is also decreasing in the same manner with increasing polymer chain length. When comparing $P4CD_{(1:1)}$, $P4CD_{(2:1)}$ and $P4CD_{(1:2)}$ hydrogels with same polymer length but differing β-CD content, it was observed that, lowest burst and highest sustained release was obtained with hydrogel $P4CD_{(2:1)}$ containing highest β-CD content. The slower release of hydrogels with higher β-CD content can be ascribed to the formation of inclusion complex of the drug with β-CD.

4.3.3. Fabrication of Patterned Hydrogels

The hydrogel system described here is ideal for obtaining micropatterns since the process is photo-chemically initiated. Spatially controllable property of thiol-ene photoaddition reaction is utilized for fabrication of micro-patterned hydrogels on a solid substrate. For this purpose, the gel precursor $P4CD_{(2:1)}$ was spin-coated on the Si/SiO_2 surface, which had been modified with methacrylate bearing trimethoxysilane for further covalent attachment of hydrogel, subsequently a photomask was placed in contact and the surface is set under UV light. After 30 min. of irradiation, photomask was taken of and unreacted species were washed away with organic solvents (Figure 4.8). Optical microscopy images revealed that thiol-ene reaction took place only illuminated region and square shaped hydrogel microstructures covalently attached on the solid substrate were obtained. These hydrogel microstructures can be expected to undergo efficient functionalization as described for the bulk hydrogels in the previous sections.

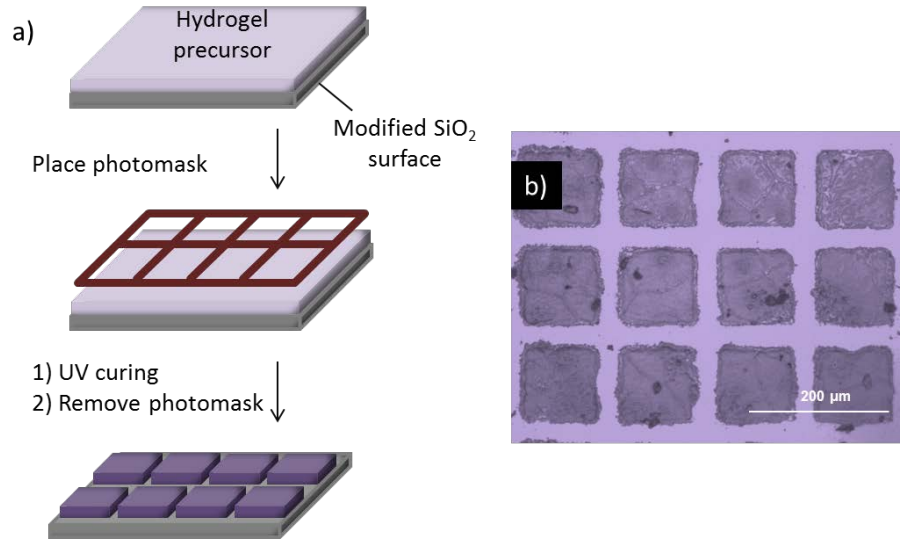


Figure 4.8. a) Fabrication of micropatterned hydrogels on modified silica surface b) Optical microscopy image of patterned hydrogel.

4.4. Conclusions

In this study, synthesis and characterization of polyethylene glycol (PEG)-based chemically cross-linked hydrogels containing discrete β-CD units were demonstrated. Hydrogels were synthesized using allyl homobifunctional linear PEGs and heptavalent thiol functionalized β-cyclodextrin crosslinker via radical induced thiol-ene click chemistry. Various hydrogels comprising different molecular weight PEGs and crosslinker feed ratio were investigated in terms of physical properties such as water uptake capacity, surface morphology and rheological behaviors. The drug sorption and controlled release studies of obtained hydrogels have been tested by employing puerarin as a model molecule. The drug uptake and release properties were shown to be depended on hydrogel composition. In addition to preparation of bulk and macroscale hydrogels, the methodology was extended to fabrication of microstructures on solid substrates. It is believed that both macroscale and micropatterned hydrogels with discrete CD units may find potential application in design of controlled drug release systems.

5. RAFT-MEDIATED TELECHELIC POLYMERS: VERSATILE PLATFORMS FOR POLYMER MODIFICATION AND CHEMICAL CROSSLINKING OF HYDROGEL NETWORKS

5.1. Introduction

Functional polymers carrying chemically bound reactive groups that can undergo post-polymerization reactions are remarkably versatile macromolecular platforms that find variety of applications in different areas [87, 127, 128]. The large interest of designing new polymers that allow post-polymerization functionalization stem from the possibility of polymer modification, attaching desired reagents onto these polymers or constructing other polymer based materials by utilizing the existing reactive groups. Polymeric scaffolds bearing strategically placed reactive groups are thus suited in material engineering and biomedical-pharmaceutical sciences to selectively bind chemical agents or to tune and modify the polymer properties [129–131].

The reactive functionalities can be present at side groups of polymer chains or they can be located at chain ends. Polymers carrying reactive functional groups at polymers ends are referred as end-functional polymers. Specific names given to end-functional polymers are semitelechelic polymers, polymers with one end group functionality; homotelechelic or simply telechelic polymers with two end group functionality in which both ends possess the same functional group and heterotelechelic polymers, polymer carrying different functional at polymer ends (Figure 5.1).

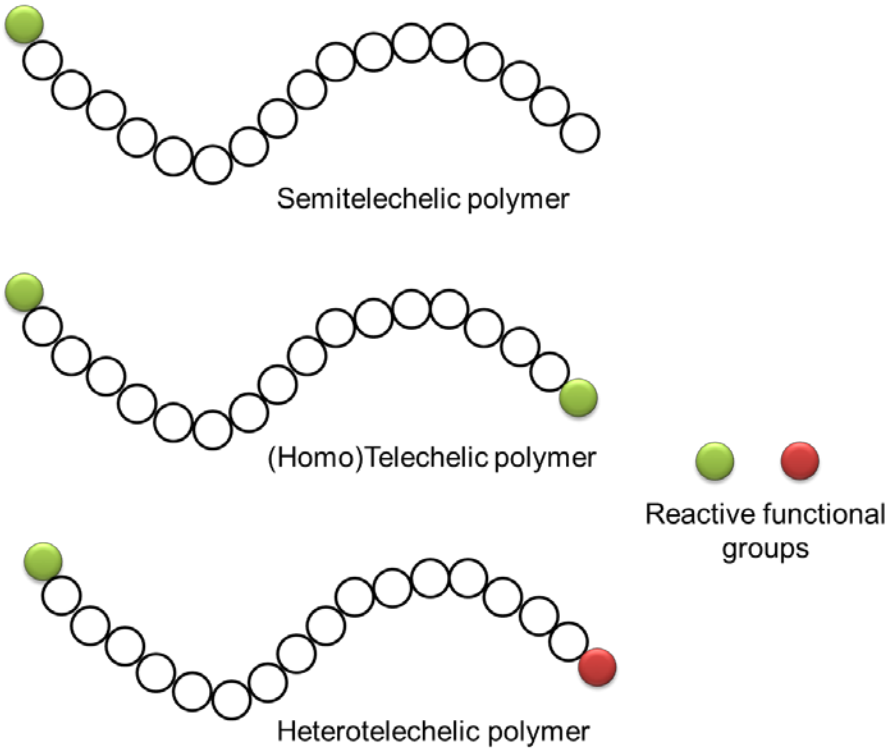


Figure 5.1. Representation of various end-functional polymers.

Polymers bearing reactive functional groups at two chain-ends, known as telechelics are important functional macromolecules used as functional supports, building blocks, crosslinkers and chain extenders [132–134]. In conventional free radical polymerization, obtaining telechelic polymers is an infeasible task; however the growing advancements in controlled radical polymerization (CRP) techniques allow one to design and synthesize end group reactive polymers with desired functionalities. For the polymers obtained by the controlled radical polymerization techniques, synthesis of telechelics is conveniently managed by utilizing various strategies. Oftentimes, such polymers are obtained by i) using a functional group containing initiator, ii) quenching the polymerization by functional group containing chain-capping reagent, or iii) chain-end modification of the polymer by post-polymerization modification reactions [86, 97, 135].

Reversible addition-fragmentation chain transfer polymerization (RAFT) is a controlled living radical polymerization technique that has been emerged as a powerful method for fabrication of diverse polymeric architectures with low polydispersity indices and pre-determined molecular weights [94]. The method possesses important advantages such as, broad functional group and solvent tolerance, compatibility for vast range of

monomers and devoid of requiring any toxic metal catalysts [95]. RAFT polymerization is also a highly efficient technique to prepare well-defined end-functional polymers [137]. In the RAFT process, reactive functional group available on the chain transfer agent (CTA) is preserved at one end of polymer chains enabling the synthesis α-end functional polymers (Figure 5.2). In some circumstances, thiocarbonylthio groups can also be processed to obtain α-ω-telechelic polymers. By implementing RAFT polymerization with functional group containing CTAs and utilizing the post-polymerization chain modification techniques, telechelic polymers with specific functionalities can be obtained [138].

Figure 5.2. General representation of a RAFT polymerization process depicting the retention of R and Z groups after polymerization.

As depicted in the previous sections, hydrogels, cross-linked hydrophilic polymer networks have been highlighted with promising potential applications. Over the past decade, various synthetic strategies have been employed for preparation of hydrogels for wide range of applications. Beside the synthesis of covalently cross-linked hydrogels via free radical crosslinking of hydrophilic polymerizable units, various 'click chemistry' methodologies including thiol-ene reactions, transition metal-mediated click reactions and Diels-Alder reactions were effectively utilized in crosslinking of monomers and polymers decorated with complementary functional groups. An important concept about hydrogel preparation is the synthesis of structurally well-defined hydrogels where the network formation is accurately controlled by using building blocks containing precisely positioned crosslinking points [100]. In these hydrogels 'ideal' network structure allows controlling the structure-final property relationship [24]. Well-defined hydrogel networks can be obtained by crosslinking of reactive group end-functionalized telechelic polymers with multivalent crosslinkers. RAFT mediated telechelic polymers with precise end group functionalities can thus be considered as ideal building blocks in the fabrication of well-defined network structures.

β-cyclodextrin is a well-known cyclic oligosaccharide with a torus shape and internal hydrophobic cavity. β-cyclodextrin along with other cyclodextrins has a remarkable ability of forming inclusion complexes with hydrophobic molecules. As it has been demonstrated throughout this thesis manuscript, β-cyclodextrin and its derivatives are extensively studied in the design of hydrogel-based materials for biomedical purposes, especially for formulating the sustained drug delivery systems. The chemical structure of β-cyclodextrin also allows it to be used as a multifunctional crosslinking reagent during hydrogel fabrication in which many cyclodextrin-containing hydrogels have been prepared following this strategy.

In this study, we report on the synthesis of cyclodextrin-containing chemically cross-linked hydrogels from telechelic polymers with hydrophilic side chains. Telechelic polymers containing maleimide or vinyl functionalities at their chain ends were synthesized and utilized towards fabrication of hydrogel networks as well as in post-polymerization functionalization with thiol-containing molecules. Hydrophilic polymers containing 2-hydroxyethyl methacrylate (HEMA) and/or di(ethylene glycol) methyl ether methacrylate (DEGMA) monomers at their backbone and thiol reactive groups at their chain ends were synthesized using reversible addition-fragmentation chain transfer (RAFT) polymerization and subsequent post-polymerization modification. A furan-protected maleimide-containing chain transfer agent (CTA) and a vinyl group-containing CTA were synthesized and used in the synthesis of α-end functional hydrophilic polymers. The ω-chain-ends of the obtained polymers were transformed into protected-maleimide or vinyl functionalities by using functionalized azo-initiators via radical induced cross-coupling reactions. For the polymers bearing protected-maleimide group at both chain termini, retro-Diels-Alder reaction was performed to unmask furan protecting group. The reactive groups at polymer ends were shown to be efficiently functionalized with thiol containing model compounds via nucleophilic and radical thiol-ene reactions. Thus obtained telechelic polymers were utilized in the fabrication of chemically cross-linked hydrogels (Figure 5.3). Hydrogels were synthesized by crosslinking of telechelic thiol-reactive polymers with a heptathiol-functionalized β-cyclodextrin crosslinker. Various hydrogels comprising different RAFT polymers were investigated in terms of physical properties such as water uptake capacity, surface morphology and rheological behaviors. β-

cyclodextrin containing hydrogels have been tested on drug sorption and controlled release by employing puerarin as a model drug molecule.

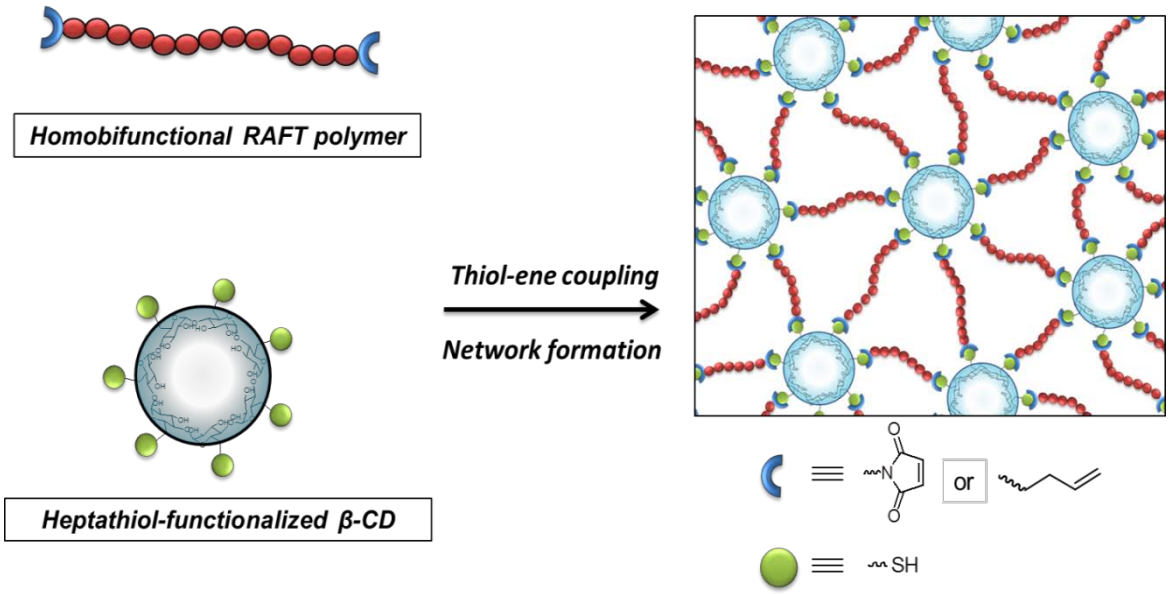


Figure 5.3. Schematic illustration of hydrogel synthesis via thiol-ene addition reactions using telechelic polymers.

5.2. Experimental

5.2.1. Materials and Characterization

4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid, 3-Buten-1-ol, 4-Dimethyl aminopyridine (DMAP), 2-Hydroxyethyl methacrylate (HEMA), Di(ethylene glycol) methyl ether methacrylate (DEGMA), 2,2′-Azobis(2-methylpropionitrile) (AIBN), 4,4'-Azobis(4-cyanovaleric acid) (ACVA), 2,2-Dimethoxy-2-phenylacetophenone (DMPA), Pentaerythritol tetrakis(3-mercaptopropionate) (PETMP), L-Glutathione-reduced and 5,5'-Dithio-*bis*-(2-nitrobenzoic acid) were purchased from Sigma-Aldrich and used as received. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was obtained from Alfa Aesar. Puerarin was obtained from TCI Chemicals. Other chemicals and solvents were purchased from Merck and used as obtained without further purification unless otherwise noted. Synthesis of furan-protected maleimide containing alcohol, exo-3a,4,7,7a-tetrahydro-2-(3-hydroxypropyl)-4,7-epoxy-1H-isoindole-1,3(2H)-dione (FM-OH) [108], 4-(3-Hydroxypropyl)-10-oxa-4-azatricyclo[5,2,1,$0^{2,6}$] dec-8-ene-3,5-dione (Azo-M) [139], cylic-RGD peptide (c(RGDfC)) [140] and thiol-functionalized β-cyclodextrin (β-CD$(SH)_7$)

[109] were conducted according to reported procedures. The gel permeation chromatography (GPC) measurements of polymers were carried out using a Shimadzu GPC analysis system with PSS WinGPC Unity software. PSS Gram (length/ID 300 mm × 8 mm, 10 µm particle size) column was calibrated with polymethyl methacrylate standards, using refractive index detector (RID-10A). DMAc was used as eluent at a flow rate of 1 mL/min at 30 ^{o}C. NMR spectra were recorded on a Varian 400-MHz spectrometer. Thiol-ene reactions were performed at 365 nm using a handheld UV lamp (Blak-Ray UVP model B-100AP/R high intensity UV lamp with a 100-watt spot bulb and 7° beam width).

5.2.2. Synthesis of Furan Protected Maleimide-Functionalized CTA (CTA-FM)

In a 10 mL round-bottom flask equipped with a stir bar, a solution of 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (200.0 mg, 0.72 mmol), furan-protected maleimide containing alcohol (FM-OH) (210.0 mg, 0.94 mmol), and DMAP (18.0 mg, 0.15 mmol) in 4 mL of CH_2Cl_2 was cooled to 0 °C under N_2. In another flask, EDC (150.0 mg, 0.78 mmol) was dissolved in 2 mL of CH_2Cl_2 and added dropwise to the reaction flask. The reaction was stirred at 0 °C for one hour and then allowed to warm to room temperature overnight. Then, the reaction medium was washed with saturated $NaHCO_3$, dried over Na_2SO_4, and evaporated to give viscous oil. The crude product was purified by column chromatography on silica with hexane and EtOAc (1:1) affording the final product as as a red oil (284.0 mg, 81 %). ^{1}H NMR ($CDCl_3$) δ (ppm): 7.87-7.93 (m, 2H); 7.52-7.59 (m, 1H); 7.35-7.44 (m, 2H); 6.50 (s, 2H); 5.25 (s, 2H); 4.07 (t, 2H, J = 6.4 Hz); 3.59 (t, 2H, J = 7.2 Hz); 2.84 (s, 2H); 2.40-2.74 (m, 4H); 1.95 (s, 3H); 1.90-1.97 (m, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 222.3, 176.1, 171.4, 144.5, 136.4, 132.9, 128.5, 126.7, 118.5, 80.9, 61.8, 47.4, 45.7, 35.6, 33.4, 29.7, 26.5, 24.1. Anal. Calcd. [$C_{24}H_{24}N_2O_5S_2$]: C, 59.48; H, 4.99; N, 5.78; S, 13.23. Found: C, 60.11; H, 5.09; N, 5.66; S, 12.88.

5.2.3. Synthesis of Vinyl-Functionalized CTA (CTA-V)

In a 10 mL round-bottom flask equipped with a stir bar, a solution of 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (200.0 mg, 0.72 mmol), 3-Buten-1-ol (68.0 mg, 0.94 mmol), and DMAP (18.0 mg, 0.15 mmol) in 4 mL of CH_2Cl_2 was cooled to 0 °C under N_2. EDC (150.0 mg, 0.78 mmol) was dissolved in 2 mL of CH_2Cl_2 and added dropwise to the reaction flask. The reaction was stirred at 0 °C for one hour and then allowed to warm to room temperature overnight. The reaction medium was then washed

with saturated $NaHCO_3$, dried over Na_2SO_4, and evaporated to give viscous oil. The crude product was purified by column chromatography on silica with hexane and EtOAc (4:1) to give final product as as a red oil (206.0 mg, 86 %). ^{1}H NMR ($CDCl_3$) δ (ppm): 7.87-7.95 (m, 2H); 7.53-7.60 (m, 1H); 7.35-7.45 (m, 2H); 5.72-5.88 (m, 1H); 5.08-5.14 (m, 2H); 4.17 (t, 2H, *J* = 8.0 Hz); 2.38-2.73 (m, 6H); 1.94 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 222.2, 171.5, 144.5, 133.7, 133.0, 128.5, 126.6, 118.5, 117.5, 64.0, 45.7, 33.4, 33.0, 29.8, 29.7, 24.1. Anal. Calcd. [$C_{17}H_{19}NO_2S_2$]: C, 61.23; H, 5.74; N, 4.20; S, 19.23. Found: C, 60.76; H, 6.04; N, 4.46; S, 20.08.

5.2.4. Synthesis of Bisvinyl-Functionalized Azo Initiator (Azo-V)

To a stirred solution of 4,4'-Azobis(4-cyanovaleric acid) (1.0 g, 3.57 mmol), 3-Buten-1-ol (670.0 mg, 9.27 mmol) and DMAP (175.0 mg, 1.43 mmol) in 20 mL of dry CH_2Cl_2 at 0 °C, a 10 mL solution of EDC (1.50 g, 7.85 mmol) was added dropwise. The reaction was stirred at 0 °C for one hour and then allowed to warm to room temperature overnight. The reaction medium was then washed with saturated $NaHCO_3$, dried over Na_2SO_4 and evaporated to give a white solid. The crude product was purified by column chromatography on silica with hexane and EtOAc (1:1) to give final product as white solid. (1.05 mg, 76 %). ^{1}H NMR ($CDCl_3$) δ (ppm): 5.71-5.82 (m, 2H); 5.06-5.13 (m, 4H); 4.16 (t, 4H, *J* = 7.9 Hz); 2.29-2.60 (m, 12H); 1.72 (s, 3H); 1.67 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 171.2, 125.9, 117.4, 71.8, 64.0, 33.1, 32.9, 29.0, 23.7. Anal. Calcd. [$C_{20}H_{28}N_4O_4$]: C, 61.84; H, 7.27; N, 14.42. Found: C, 61.14; H, 7.94; N, 13.77.

5.2.5. Typical Procedure for RAFT Homopolymerization / Copolymerization of DEGMA and HEMA Monomers with CTA-FM and CTA-V

Polymerization procedure for copolymers P-FM2 and P-V2: DEGMA (132.0 mg, 0.70 mmol), HEMA (91.0 mg, 0.70 mmol), CTA (0.047 mmol, 22.90 mg for CTA-FM and 15.70 mg for CTA-V) and AIBN (0.77 mg, 0.0047 mmol) were dissolved in DMF (1.40 mL) and placed in a sealed round-bottom flask equipped with a magnetic stir bar. The reaction mixture was purged with nitrogen for 15 minutes and stirred at 70 °C for 12 h. After polymerization, the solvent was removed under reduced pressure and the residue was re-dissolved in minimum amount of methanol before precipitating into the cold ether. The precipitated polymer was dried under vacuum and characterized using SEC and ^{1}H NMR.

5.2.6. Radical Cross-Coupling of Thiocarbonylthio End-Functional Polymers with Functionalized Azo Initiators

Synthesis of polymer P-BFM2: Polymer P-FM2 (100.0 mg, 0.028 mmol) and Azo-M (480.0 mg, 0.70 mmol) were dissolved in DMF (4.0 mL) in a sealed tube. The reaction mixture was purged with nitrogen for 15 minutes and stirred at 70 °C for 4 h. After the reaction, the solvent was removed in vacuo and the resulting polymer P-BFM2 was purified by dialysis (1:1 EtOAc : MeOH, MWCO 6-1000 Da).

Synthesis of polymer P-BV2: Polymer P-V2 (100.0 mg, 0.026 mmol) and Azo-V (257.0 mg, 0.66 mmol) were dissolved in DMF (4 mL) in a sealed tube. The mixture was purged with nitrogen for 15 minutes and stirred at 70 °C for 4 h. Then, the solvent was removed in vacuo and the resulting polymer P-BV2 was purified by precipitating into the cold ether (repeated twice).

Synthesis of polymer P-FMV2: Polymer P-FM2 (100.0 mg, 0.028 mmol) and Azo-V (272.0 mg, 0.70 mmol) were dissolved in DMF (4.0 mL) in a sealed tube. The reaction mixture was purged with nitrogen for 15 minutes and stirred at 70 °C for 4 h. After the reaction, the solvent was removed in vacuo and the resulting polymer P-FMV2 was purified by precipitating into the cold ether (repeated twice).

5.2.7. Activation of Protected-Maleimide Functional Groups

Polymer P-BFM2 (50 mg, 0.014 mmol) or P-FMV2 (50 mg, 0.015 mmol) were dissolved in MeOH (30 mL) in a 100 mL round-bottom flask. The solvent was removed to form a thin film of polymer on the flask surface. The polymers were heated under vacuum at 110 ^{o}C for 8 h to afford telechelic polymers P-BM2 or P-MV2, quantitatively.

5.2.8. Functionalization of Maleimide Homotelechelic Polymer P-BM2 with Glutathione

P-BM2 (50.0 mg, 0.014 mmol) was dissolved in DMF (0.4 mL) and degassed for 10 minutes. Glutathione (10.0 mg, 0.034 mmol) and triethylamine (0.1 eq. per thiols) was then added to this solution and the reaction mixture was stirred at room temperature for 12 h. After the reaction, the obtained polymer was purified by dialysis against acetonitrile.

5.2.9. Functionalization of Vinyl Homotelechelic Polymer P-BV2 with Glutathione

P-BV2 (50.0 mg, 0.013 mmol), glutathione (15.0 mg, 0.050 mmol) and DMPA (0.2 eq. per thiols) was placed in a vial and dissolved in DMF (0.15 mL). The solution was degassed for 10 minutes and then UV irradiated for 30 minutes at 365 nm. The obtained polymer was purified by dialysis against acetonitrile.

5.2.10. Functionalization of Maleimide-Vinyl Heterotelechelic Polymer P-MV2 with Thiol-Functionalized cRGD and Glutathione

P-MV2 (50.0 mg, 0.014 mmol) was dissolved in DMF (0.4 mL) and degassed for 10 minutes. c(RGDfC)-thiol (10.0 mg, 0.017 mmol) and triethylamine (0.1 eq. per thiols) was then added to this solution. The reaction mixture was stirred at room temperature for 12 h and the resulting polymer was purified by dialysis against DMSO and acetonitrile to obtain functionalized polymer P-FV2.

Polymer P-FV2 (0.014 mmol), glutathione (7.5 mg, 0.025 mmol) and DMPA (0.2 eq. per thiols) was placed in a vial and dissolved in DMF (0.15 mL). After degassing solution for 10 min., the reaction mixture was UV irradiated for 30 minutes at 365 nm. The purified polymer P-FF2 was obtained by dialysis against acetonitrile.

5.2.11. Representative Hydrogel Formation via Nucleophilic Thiol-Ene Reaction (Synthesis of Hydrogels H-CM(1-3))

Hydrogels were obtained through multiple thiol-ene reactions between maleimide groups of polymers and thiol-functionalized β-cyclodextrin (β-CD$(SH)_7$). For a representative example, bismaleimide-functionalized polymer P-BM2 (50.0 mg, 14.0 x10^{-3} mmol) was placed in a vial and dissolved in DMF (100 µL). A solution of β-CD$(SH)_7$ (5.0 mg, 4.0 x10^{-3} mmol) and triethylamine (0.39 µL, 2.8 x10^{-3} mmol) in DMF (100 µL) was then added to this solution. In order to achieve homogenous gelation the mixture was sonicated briefly. In about one minute, no flow of sample was observed. In order to ensure the complete conjugation, gelation was continued for 12 h. After hydrogel formation, unreacted reagents were removed by washing the gel with DMF followed by distilled water several times. Obtained gel sample was frozen and lyophilized to yield dried hydrogel.

5.2.12. Representative Hydrogel Formation via Radical Thiol-Ene Reaction (Synthesis of Hydrogels H-CV(1-3))

Hydrogels synthesis was accomplished through multiple thiol-ene reactions between vinyl groups of homotelechelic polymers and β-CD(SH)$_7$ crosslinker. Bisvinyl-functionalized polymer P-BV2 (50.0 mg, 0.013 mmol) was placed in a vial and dissolved in DMF (100 µL). A mixture of β-CD(SH)$_7$ (4.72 mg, 3.8 x10^{-3} mmol) and DMPA (0.2 eq. per thiols) in DMF (100 µL) was then added to this solution. The mixture was irradiated with UV light for 30 minutes at 365 nm. After hydrogel formation, unreacted species were removed by washing the hydrogel with DMF and distilled water several times. Dried hydrogel was obtained by freeze-drying the swollen gel sample.

5.2.13. Hydrogel Formation By Using Tetrathiol-functionalized Crosslinker PETMP (Synthesis of Hydrogels H-CM4 and H-CA4)

Hydrogel H-CM4: In a vial, P-BM3 (50.0 mg, 12.5 x10^{-3} mmol) was placed and dissolved in DMF (100 µl). A solution of PETMP (3.07 mg, 6.28 x10^{-3} mmol) and triethylamine (0.010 µl, 2.5 x10^{-3} mmol) in DMF (100 µL) was then added to this solution. The mixture was sonicated briefly. Hydrogel formation was rapid and in about half of a minute there was no flow of sample. Gelation was continued for 12 h to ensure complete conjugation. After hydrogel formation, unreacted reagents were removed by washing the gel with DMF followed by distilled water several times. Obtained gel sample was frozen and lyophilized to yield dried hydrogel.

Hydrogel H-CA4: P-BA3 (50.0 mg, 13.6 x10^{-3} mmol) was placed in a vial and dissolved in DMF (100 µL). A mixture of PETMP (3.32 mg, 6.8 x10^{-3} mmol) and DMPA (0.2 eq. per thiols) in DMF (100 µL) was then added to this solution. The mixture was irradiated with UV light for 30 minutes at 365 nm. After hydrogel formation, unreacted species were removed by washing the hydrogel with DMF and distilled water several times. Dried hydrogel was obtained by freeze-drying the swollen gel sample.

5.2.14. Determination of Thiol Content

Total thiol contents of hydrogels were determined using Ellman's method [112]. After reacting free thiol groups of hydrogels with 5,5'-Dithio-bis-(2-nitrobenzoic acid), they were quantitated by reference to the extinction coefficient of TNB^{2-} (2-nitro-5-

thiobenzoic acid) ion. Experimental procedure was briefly as follows: 0.1 M sodium phosphate, containing 1 mM EDTA (pH 8.0) buffer was prepared as reaction medium. A solution of Ellman's reagent was prepared by dissolving 4 mg 5,5'-Dithio-*bis*-(2-nitrobenzoic acid) in 1 mL of reaction buffer. 5 mg freshly prepared hydrogel sample was placed in a vial and 2.5 mL of reaction buffer and corresponding Ellman's reagent solution was added onto the sample. The mixture was incubated at 37 °C for 2 hours. Total thiol content was calculated by measuring the absorbance at 412 nm and using the molar extinction coefficient of TNB^{2-} (14,150 $M^{-1}cm^{-1}$).

5.2.15. Swelling Studies

A sample of hydrogel was placed in a vial containing distilled/deionized water at room temperature and the change in mass of sample was followed as a function of time till the hydrogel showed constant weight. The weight change percentage was obtained from the equation:

$$\% \text{ Swelling} = (W_{wet} - W_{dry}) / W_{dry} \times 100,$$

where W_{wet} and W_{dry} refer to the wet and dry hydrogel weights, respectively. The swelling curves were plotted by taking the average of three measurements.

5.2.16. Scanning Electron Microscopy (SEM)

The surface morphologies of dried hydrogels were analyzed with scanning electron microscopy (SEM) using an ESEM-FEG/EDAX Philips XL-30 (Philips, Eindhoven, The Netherlands) instrument with an accelerating voltage of 10 kV.

5.2.17. Rheological Measurements

The hydrogel samples prepared as disks were characterized in terms of rheological behaviors. Loss (G″) and storage (G′) moduli of the swollen hydrogels were evaluated in triplicate by measuring the angular frequency dependence of modulus values. Tests were carried out at 25 ^{o}C by applying 0.5 % strain between 0.1–100 rad/s using an Anton Paar MCR 302 rheometer. A parallel plate of 8 mm diameter was used for analysis and the gap between plates was adjusted to 2.0 mm.

5.2.18. Drug Loading and Release Studies

Drug uptake and release properties of hydrogels were evaluated using a poorly water-soluble drug puerarin. Loading the drug into the hydrogels was accomplished by a solution absorption method. Hydrogel samples (~50 mg) were soaked in 25 mL 0.80 mg/mL puerarin solution at 37 ^{o}C. Soaking solution was gently vibrated at 100 rpm to increase the loading efficiency. Puerarin concentration in the soaking solution was analyzed using a UV-vis spectrophotometer at 250 nm till a constant concentration was achieved. Studies showed that for loading time a 24 h period was sufficient. The drug loading amount of the hydrogel was determined from the initial and final concentration of soaking medium. The percent drug loading was calculated as:

$$\% \text{ Loading} = W_{drug} / (W_{drug} + W_{hydrogel}) \times 100,$$

where W_{drug} and $W_{hydrogel}$ refer to the weight of the drug and weight of the hydrogel sample, respectively.

Drug loaded hydrogels were roughly rinsed with distilled water (2 mL) and added to 15 mL distilled water as the release medium. The release tests were carried out at 37 ^{o}C by shaking the medium at 100 rpm. 5 mL of release medium was taken out from release medium with predetermined time intervals and replaced with same volume of fresh water. The amount of drug in collected media was determined spectrophotometrically. The results were expressed in terms of cumulative release as a function of time.

5.3. Results and Discussion

5.3.1. Synthesis of Telechelic Maleimide and Vinyl-Functionalized Polymers

In order to obtain homotelechelic polymers with maleimide and vinyl functionalities, a series of synthesis and post-polymerization modification steps were carried out. Firstly, chain transfer agents carrying protected-maleimide and vinyl groups were synthesized and utilized in the RAFT polymerization of two hydrophilic monomers HEMA and DEGMA. Subsequently, radical induced cross-coupling reactions with functionalized azo-initiators were performed to introduce functional groups to the polymer chain ends.

Reversible addition-fragmentation chain transfer (RAFT) polymerization is one of the most versatile controlled radical polymerization technique that is suited in the polymerization of various kinds of monomers with narrow molecular weight distributions.

In the design and synthesis telechelic polymers, RAFT polymerization was explored as a convenient method to introduce desired functional groups at the α and ω-ends of the polymers. The main component in the RAFT procedure is chain transfer agent (CTA), typically a thiocarbonylthio compound responsible for the controlled growth of the polymerization. The functional group present on the CTA (R group) is retained at the one chain end of the polymer enabling the synthesis of α-functional polymers. The RAFT group (thiocarbonylthio and Z group) can also be further manipulated thorough various methods to introduce desired functionality at ω-end. To install protected-maleimide and vinyl groups at one chain end, CTAs bearing these groups were synthesized (CTA-FM and CTA-V, Figure 5.4 D). RAFT polymerization of HEMA and DEGMA monomers were carried out with CTA-FM and CTA-V chain transfer agents to install furan-protected maleimide and vinyl functional groups respectively at α-end of polymers. (Figure 5.4 A,B). RAFT homopolymerization/copolymerization reactions of the monomers were conducted in dimethylformamide at 70 ^{o}C (Table 5.1, Items 1-8). For the polymers obtained by CTA-FM, the temperature was low enough to avoid any retro Diels-Alder reaction that might mediate deprotection of the masked maleimide groups. After completing the polymerization reactions, DMF was removed in vacuo and the polymers were purified upon their precipitation with diethyl ether. Size exclusion chromatography demonstrated that polymers were obtained with good control over molecular weight and relatively narrow polydispersities (Table 5.1).

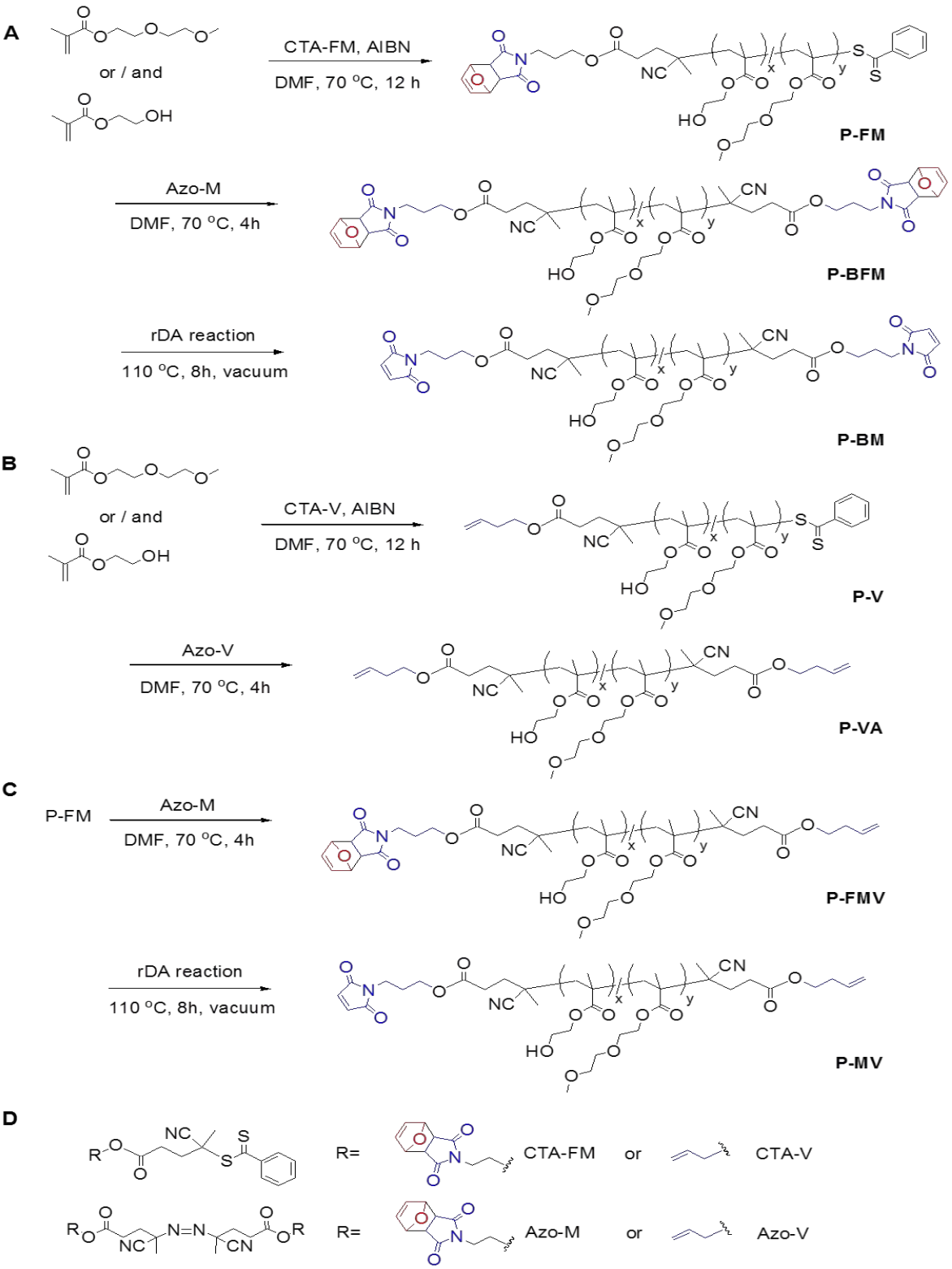


Figure 5.4. Synthesis of A, B) maleimide and vinyl-functionalized homotelechelic and C) maleimide-vinyl-functionalized heterotelechelic raft polymers, D) functional group-containing CTAs and azo compounds used in this work.

Table 5.3. Conversion, molar mass, and polydispersity data for the RAFT mediated polymers.

Entry	Polymer[a]	$F_{theo.}$[b]	$F_{cal.}$[c]	% Yield	$M_{n,\ theo.}$	$M_{n,\ NMR}$[c]	$M_{n,\ GPC}$[d]	M_w/M_n[d]
1	P-FM1	1 : 0	-	76.0	4780	4250	3860	1.27
2	P-FM2	1 : 1	1 : 1.32	71.0	3870	4015	3590	1.29
3	P-FM3	0 : 1	-	88.0	3930	3840	3350	1.32
4	P-V1	1 : 0	-	72.0	4400	3720	3460	1.26
5	P-V2	1 : 1	1 : 1.37	77.0	4010	3640	3550	1.31
6	P-V3	0 : 1	-	85.0	3660	3590	3220	1.32

[a] $[M]_o/[CTA]/[AIBN]$: 30/1/0.1; $[M]_o$: 1 M; CTA: CTA-FM for P-FM(1-3) and CTA-V for P-V(1-3); Temp.: 70 °C; Time: 12 h; Solvent: DMF. [b] $F_{theo.}$= [DEGMA]:[HEMA]. [c] $F_{cal.}$= [DEGMA]:[HEMA], determined by ^{1}H NMR. [d] Estimated by SEC eluted with DMAc using poly(methyl methacrylate) standards.

Analysis of these polymers using ^{1}H NMR revealed successful incorporation of the furan-protected maleimide and alkene functionalities at one chain end of polymers. In HEMA/DEGMA copolymer synthesized by protected-maleimide containing CTA-FM, the proton resonances around 6.55, 5.12 and 2.92 ppm are due to the bicyclic moiety of the CTA (Figure 5.5a). The presence of the RAFT group at the other chain end was also established by proton resonances belonging to the phenyl ring in the 7.83-7.45 ppm region. For the HEMA/DEGMA copolymer synthesized by vinyl-functionalized CTA-V, alkene proton resonances were observed at 5.05-5.13 and 5.84-5.73 regions (Figure 5.6a). Likewise, phenyl ring proton resonances at aromatic region indicate the presence of the RAFT group at chain termini.

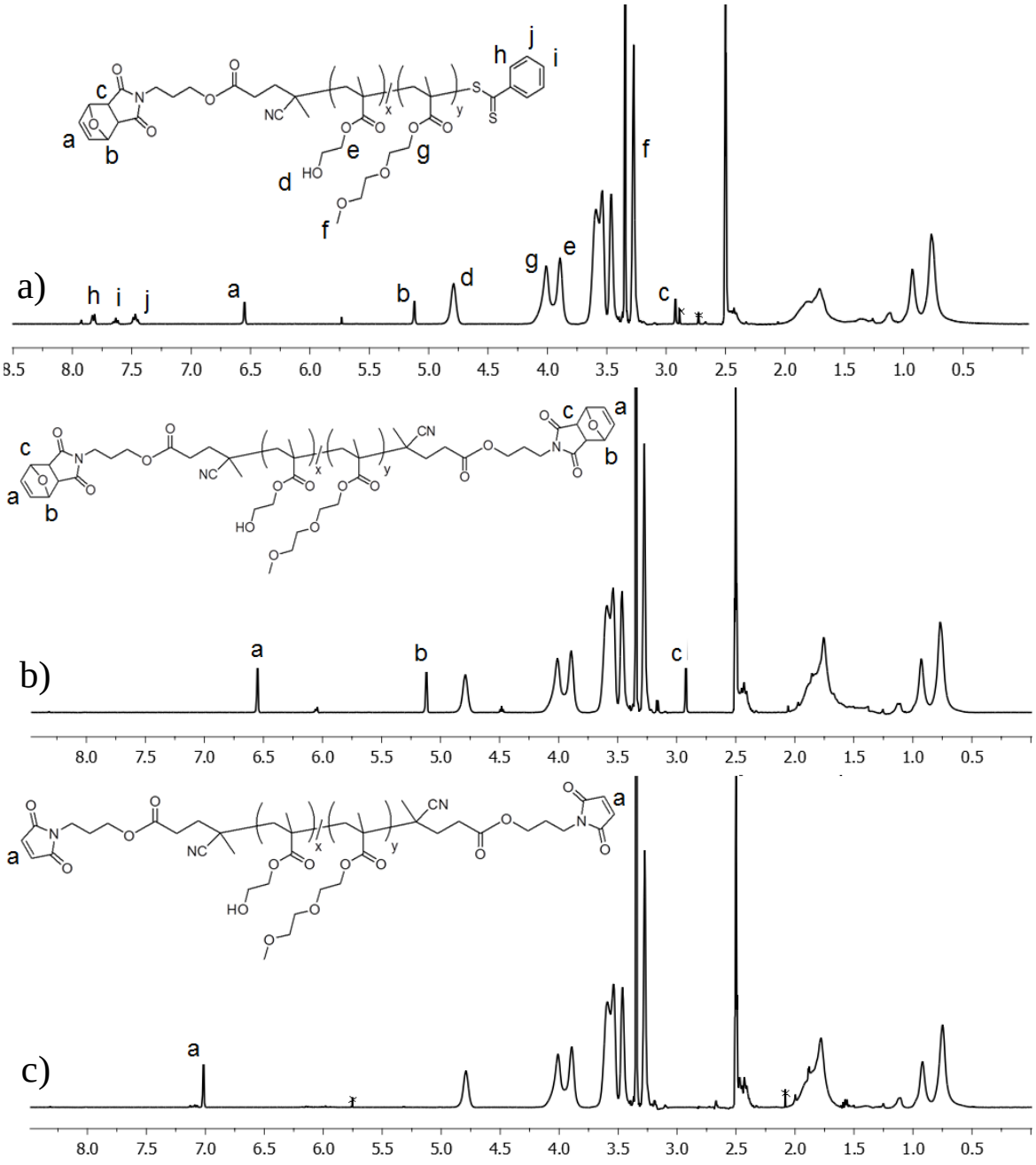


Figure 5.5. ^{1}H NMR spectra of polymers a) P-FM2 b) P-BFM2 and c) P-BM2 (in *d*-DMSO).

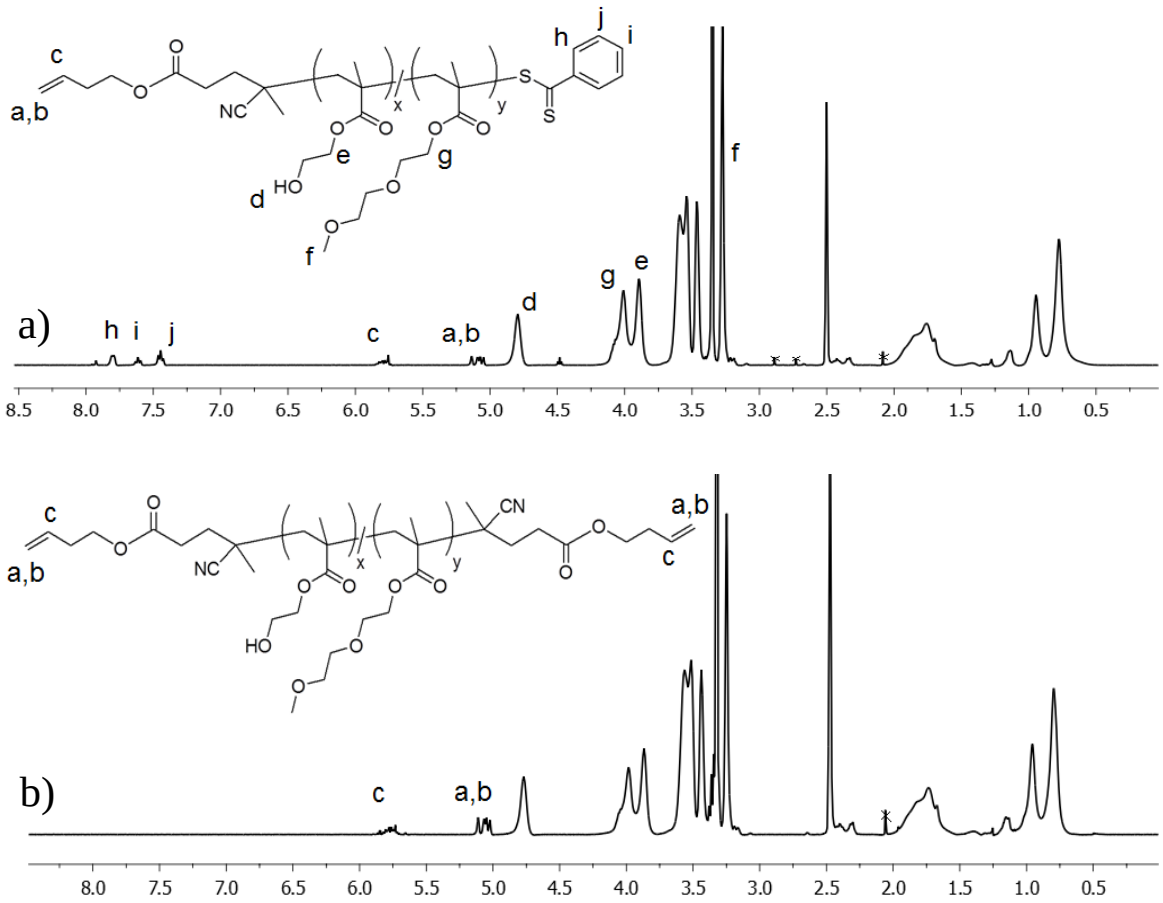


Figure 5.6. ^{1}H NMR spectra of polymers a) P-V2 and b) P-BV2.

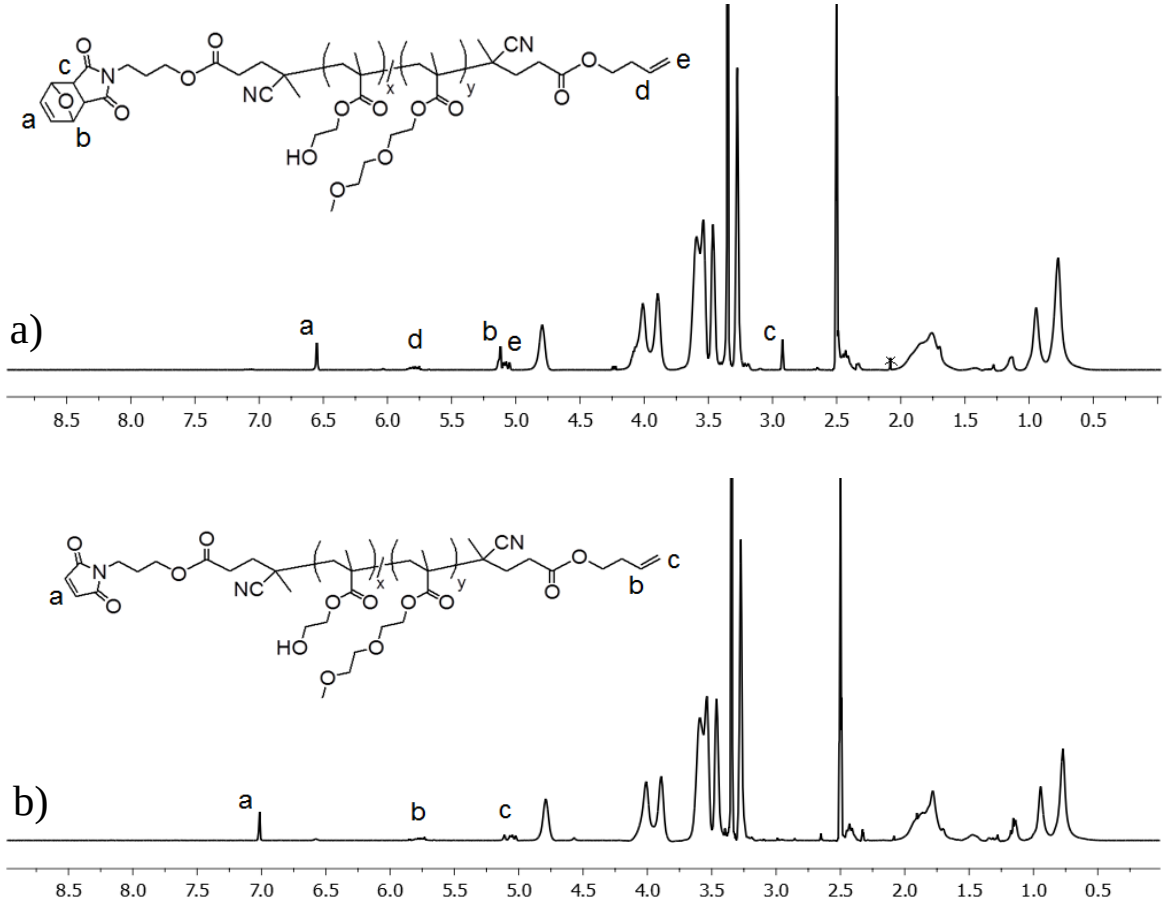


Figure 5.7. ^{1}H NMR spectra of polymers a) P-FMV2 and b) P-MV2.

The thiocarbonylthio groups present at the *ω*-chain-ends of the obtained polymers were transformed into protected-maleimide or vinyl functionalities by using functionalized azo-initiators via radical cross-coupling reactions (Figure 5.4 A,B,C). Reactions were performed in DMF for 4h and in order to achieve complete transformation of end-groups, 25-fold excess functionalized azo compounds were used. After completing the reactions, polymer purifications were performed by dialysis and precipitation procedures. Thus obtained polymers were analyzed using ^{1}H NMR and it was observed that, phenyl protons of thiocarbonylthio groups were disappeared indicating the successful transformation of the end-groups (Figure 5.5b, Figure 5.6b and Figure 5.7a). The transformation was also investigated by UV-Vis analysis via following the disappearance of the characteristic absorbance of thiocarbonylthio groups (centered at λ= 302 nm) from polymer ends (Figure 5.8).

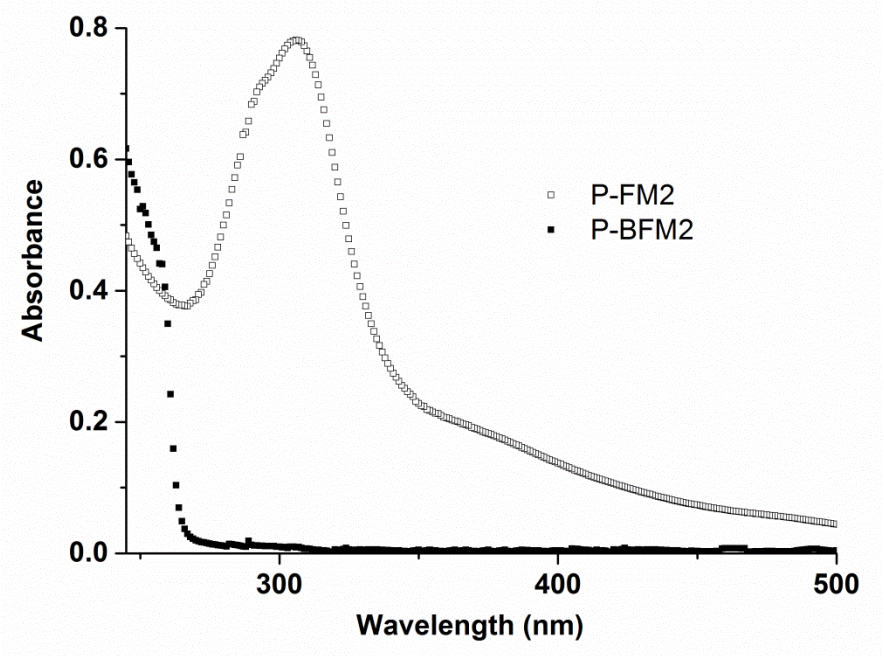


Figure 5.8. Representative UV-vis spectra of copolymers P-FM2 and P-BFM2 (after end-group modification).

Polymers bearing protected-maleimide end groups were subjected to retro Diels-Alder reaction to remove protecting furan moiety and obtain the maleimide units in their thiol-reactive form. Deprotection was performed by heating polymers in vacuo at 110 ^{o}C. ^{1}H NMR analysis of HEMA/DEGMA copolymer indicated quantitative unmasking of maleimide groups by disappearance of the peaks at 6.55, 5.12 and 2.92 ppm and formation of a new peak at 7.02 ppm due to the maleimide group (Figure 5.5c).

5.3.2. Functionalization of Thiol Reactive Homo and Hetero-telechelic Polymers

The post-polymerization functionalization efficiencies of polymers containing maleimide or vinyl groups were demonstrated on HEMA/DEGMA copolymers (Figure 5.9). For the copolymer P-BM2 carrying maleimide groups at chain ends, thiol conjugation was studied using a thiol-containing tripeptide glutathione. The nucleophilic thiol-ene addition reaction was carried out in DMF at room temperature and catalytic amount of triethylamine was used to accelerate reaction. After the reaction, residual unbound glutathione was removed by dialysis in acetonitrile to obtain functionalized polymer in pure form. According to ^{1}H NMR analysis, complete thiol conjugation was observed due to the disappearance of maleimide signals at 7.01 ppm and formation of new proton resonances at 2.18, 2.89 and 4.62 due to the glutathione moiety (Figure 5.10a).

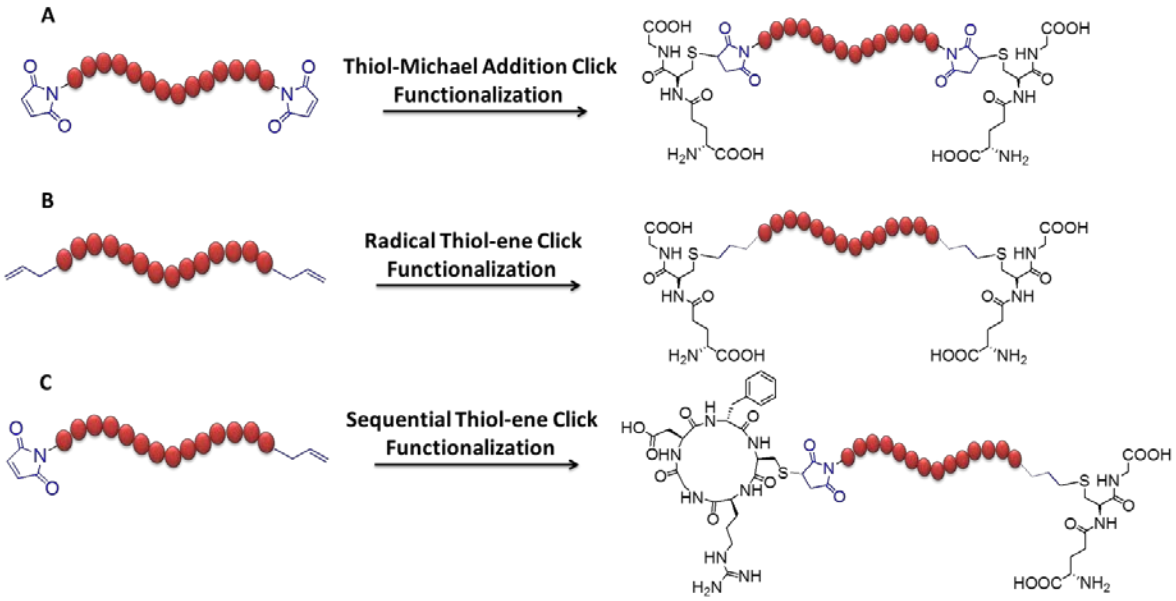


Figure 5.9. A,B,C) General representation of post-polymerization functionalization reactions for RAFT polymers carrying maleimide and vinyl end groups.

The copolymer P-BV2 carrying vinyl end groups were functionalized with glutathione by employing a radical thiol-ene addition. The reaction was performed in presence of DMPA as a photoinitiator under UV excitation (365 nm). After completing the reaction, the crude polymer was purified by dialysis against acetonitrile. Analysis of obtained polymer with ^{1}H NMR revealed complete disappearance of double bond peaks around 5.06 and 5.78 ppm along with the signals arising from glutathione moieties (Figure 5.10b).

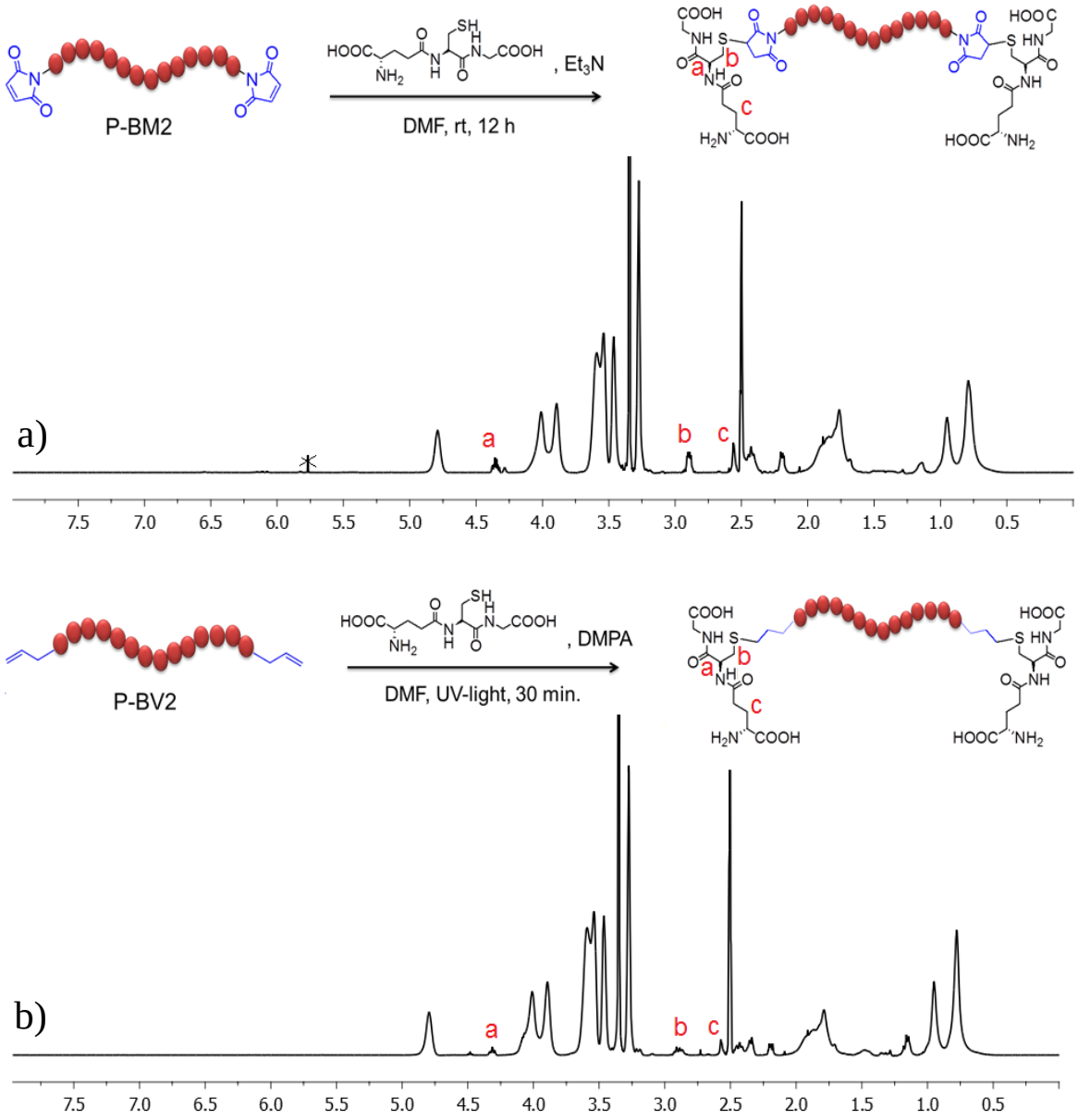


Figure 5.10. Functionalization and ^{1}H NMR characterization of maleimide a) and vinyl b) end-functional RAFT copolymers with glutathione.

Selective stepwise functionalization of the copolymer P-MV2 carrying maleimide and vinyl end-functionalities was investigated by reacting it with thiol-functionalized cyclic peptide c(RGDfC), followed by glutathione. In the ^{1}H NMR analysis, disappearance of the proton resonance at 7.01 ppm and the emergence of a new peak around 7.35 ppm belonging to the aromatic protons of cyclic peptide implied successful attachment of the c(RGDfC), via the Michael addition (Figure 5.11). Further reaction of this copolymer with

glutathione resulted in the complete disappearance of the proton resonances corresponding to the double bond signals around 5.06 and 5.78 ppm. Formation of new signals belonging to glutathione also showed the successful functionalization via radical thiol-ene reaction.

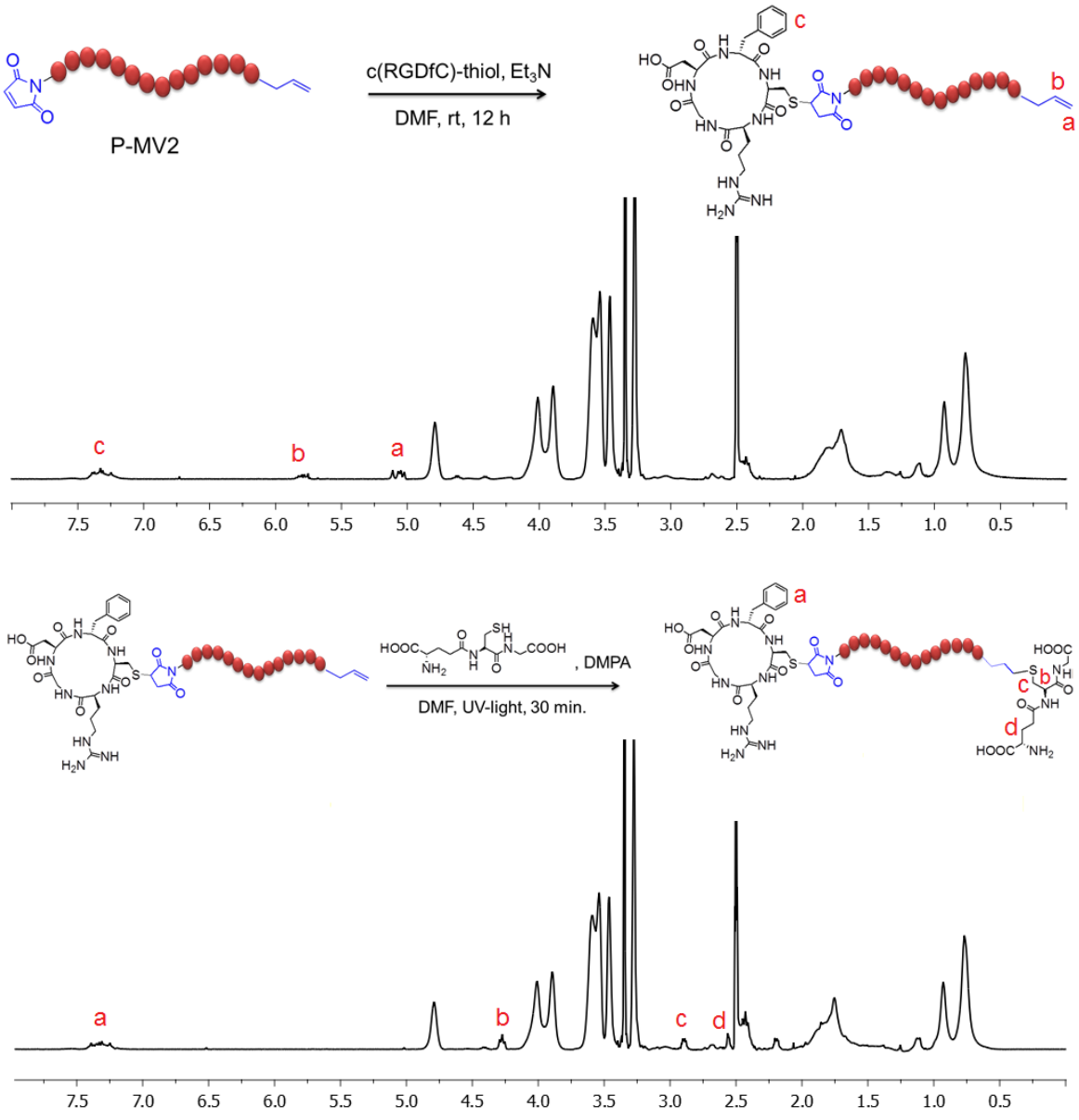


Figure 5.11. Sequential thiol-ene functionalization of maleimide-vinyl end-functional RAFT copolymer with c(RGDfC) and glutathione.

5.3.3. Synthesis and Characterization of Hydrogels

The obtained homobifunctional polymers were evaluated in fabrication of hydrogel networks. Hydrogels were prepared via thiol-ene reactions of bismaleimide or bisvinyl-

functionalized RAFT polymers with a heptavalent thiol-functionalized β-cyclodextrin crosslinker (β-CD(SH)$_7$). Hydrogels via nucleophilic thiol-ene reactions were obtained by mixing the solutions of maleimide-functionalized homotelechelic polymers and β-CD(SH)$_7$. Through multiple Michael additions of thiol groups onto maleimides, rapid crosslinking occurs and in approximately one minute, no flow of sample was observed. In order to ensure the complete network formation, gelation was continued for 12 h. Gel formation is promoted by using catalytic amount of Et_3N. For the bisvinyl-functionalized RAFT polymers, hydrogels were obtained via photo-initiated radical thiol-ene reaction of vinyl functionalities with thiol groups of β-CD(SH)$_7$ crosslinker. The reactions were carried out under UV irradiation (365 nm) and as photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DMPA) was used. In order to compare the effect of monomer type on physical and morphological properties, a library of hydrogels were prepared using HEMA/DEGMA based homopolymers and copolymers. Table 5.2 summarizes the compositions, feed ratios and gel conversions of the obtained hydrogels

Table 5.2. Gel conversions, total thiol contents and drug loading amounts of synthesized hydrogels.

Entry	Hydrogel	Polymer	Feed Ratio [-SH]:[alk.]	Gel Conv. %	Thiol Content[a] (mmol x 10^{-4})	% Thiol Cons.	Drug Load[b] (mg/g dry gel)
1	H-CM1	P-BM1	1 : 1	84.0	21.7 / 3.95 (±0.41)	81.8	19.07 ± 3.41
2	H-CM2	P-BM2	1 : 1	87.0	22.9 / 3.82 (±0.38)	83.3	23.84 ± 2.73
3	H-CM3	P-BM3	1 : 1	89.0	23.8 / 3.38 (±0.32)	85.8	27.59 ± 4.31
4	H-CV1	P-BV1	1 : 1	81.0	24.5 / 5.75 (±0.53)	76.5	15.31 ± 2.42
5	H-CV2	P-BV2	1 : 1	79.0	25.0 / 6.35 (±0.47)	74.6	20.29 ± 3.56
6	H-CV3	P-BV3	1 : 1	83.0	25.3 / 4.98 (±0.43)	80.3	22.17 ± 3.19

[a] Thiol amount used for synthesis of 5 mg hydrogel / Determined thiol amount in 5 mg hydrogel sample (Data in triplicate). [b] Drug loading of hydrogels in 0.80 mg/mL soaking solution of puerarin (Data in triplicate).

To demonstrate the versatility of designing homobifunctional RAFT polymers with desired side chain segments and subsequent use in hydrogel network formation, HEMA/DEGMA based polymers with maleimide and vinyl end-functionalities were utilized in gel fabrication. Thiol-maleimide Michael addition and radical thiol-ene reactions have attracted much attention recently to fabricate and functionalize polymeric

materials since the reactions proceed under metal-free conditions with high conversions. Homotelechelic RAFT polymers with maleimide and alkene end-functionalities were reacted with thiol-modified β-CD(SH)$_7$ crosslinker through nucleophilic and radical thiol-ene reactions to prepare hydrogels H-CM(1-3) and H-CV(1-3) (Table 5.2, Entry 1-6). HEMA/DEGMA based polymers with close molecular weights were used in gel fabrication and in each case; the feed ratio of polymer and crosslinker was adjusted to one to one coupling of alkene groups with thiols. In ideal circumstances, it can be assumed the complete pairing of maleimide or vinyl groups with thiols that would allow the formation of well-defined network structures. On the other hand, considering the steric bulk of polymer chains, chain entanglements, loop formations, side reactions and steric crowding around the crosslinking sites, ideal network formation would be diminished. Hydrogels were obtained with moderately good gel conversions yet attempts of further increase in yields were unsuccessful and this was attributed to abovementioned reasons. Relatively higher gel conversions were obtained for the hydrogels obtained by thiol-maleimide addition reaction.

In order to gather information about the crosslinking efficiency of the processes, residual thiol-group content of hydrogels were undertaken via Ellman's analysis. Hydrogels prepared using various homobifunctional RAFT polymers were treated with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) and the resulting 2-nitro-5-thiobenzoic acid (TNB) species were quantified spectrophotometrically. In order to minimize the disulfide formation of thiol groups, hydrogels were freshly prepared and analyzed. The values of thiol amount used in hydrogel formation, determined ratio and percent thiol consumption of hydrogels are reported in Table 5.2. According to results, 80-90 % thiol consumptions of hydrogels were shown to be slightly higher for thiol-maleimide addition hydrogels. The reason for relatively low thiol consumptions were thought to be primarily the high steric crowding around the heptavalent crosslinker that diminishes the efficient coupling of complementary groups. Thus, in order to test the effect of crosslinker functional group valency on conjugation efficiency of maleimide and vinyl groups with thiols, a series of hydrogels were synthesized and analyzed with a tetra-thiol functionalized crosslinker (Figure 5.12). Hydrogels synthesized by using P-BM3 and P-BV3 polymers with tetra thiol-functionalized crosslinker resulted higher gel conversions (% 97 and % 91, respectively) and percent thiol consumptions (% 91.6 and % 86.3,

respectively) that show the effect of steric crowding around the crosslinking points on conjugation efficiency (Table 5.3). Another reason for presence of left over thiol groups was attributed to the presence of a small percentage of chains initiated by AIBN during RAFT polymerization causing unequal stoichiometric balance between thiols and alkene groups.

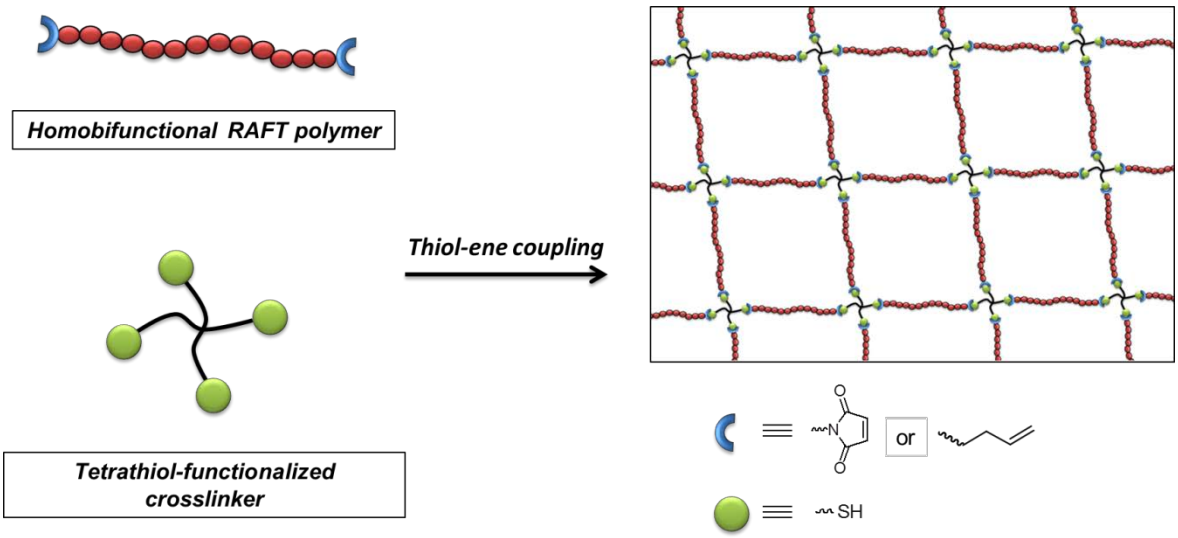


Figure 5.12. Schematic illustration of hydrogel formation using a tetrafunctional crosslinker.

Table 5.3. Gel conversions and total thiol contents of hydrogel synthesized by tetrathiol-functionalized crosslinker.

Entry	Hydrogel	Polymer	Feed Ratio [-SH]:[alkene]	% Gel Conv.	Thiol Content[a] (mmol x 10^{-4})	% Thiol Consumption
1	H-CM4	P-BM3	1 : 1	97.0	24.5 / 2.06 (±0.37)	91.6
2	H-CA4	P-BA3	1 : 1	91.0	26.0 / 3.56 (±0.44)	86.3

[a] Thiol amount used for synthesis of 5 mg hydrogel / Determined thiol amount in 5 mg hydrogel sample (Data in triplicate).

The hydrogels obtained both nucleophilic and radical thiol-ene reactions were clear and transparent samples. Based on the SEM analysis of freeze-dried hydrogels, continuous gel structures with low porosities were observed (Figure 5.13). For both of the hydrogel series prepared by bismaleimide and bisvinyl-functionalized polymers, relatively higher porous structures were observed with HEMA-based gel networks.

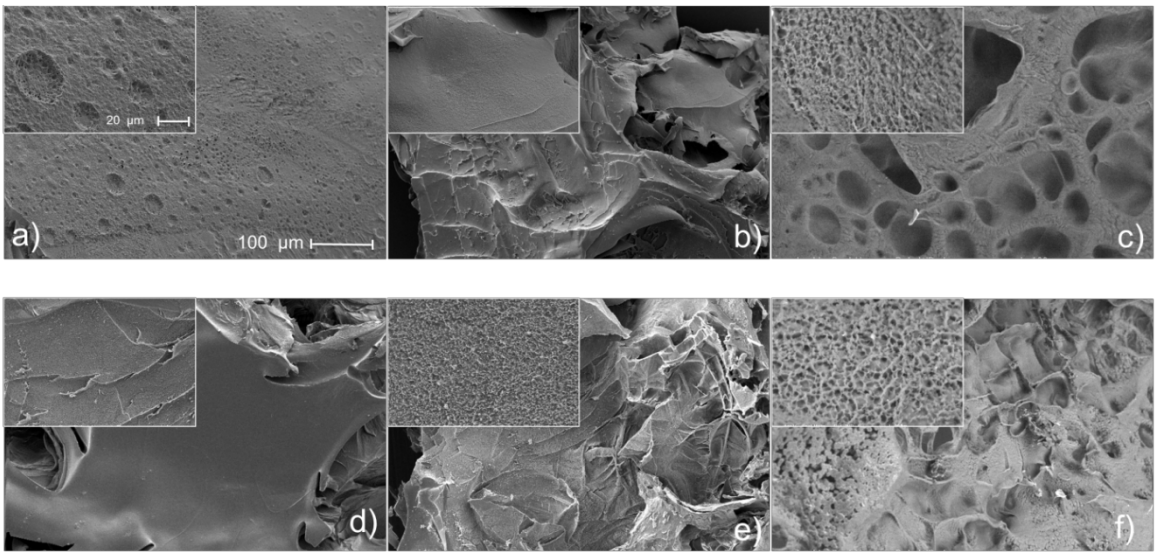


Figure 5.13. Representative ESEM images of freeze-dried hydrogels: a) H-CM1 b) H-CM2 c) H-CM3 d) H-CV1 e) H-CV2 f) H-CV3. Large image scale bar: 100 µm; small images: 20 µm.

Hydrogels were investigated in terms of swelling behaviors by recording the water uptake in pre-determined time intervals until a constant weight is attained. All hydrogels showed good swelling capacity and fast kinetics due to the presence of hydrophilic monomers in polymer backbones (Figure 5.14). It was found that, swelling properties of hydrogels exhibit dependency on polymer structure. For both type of reaction methodologies applied, higher swelling ratios were obtained with HEMA-based hydrogels. This was attributed to the higher hydrophilic character of HEMA monomer.

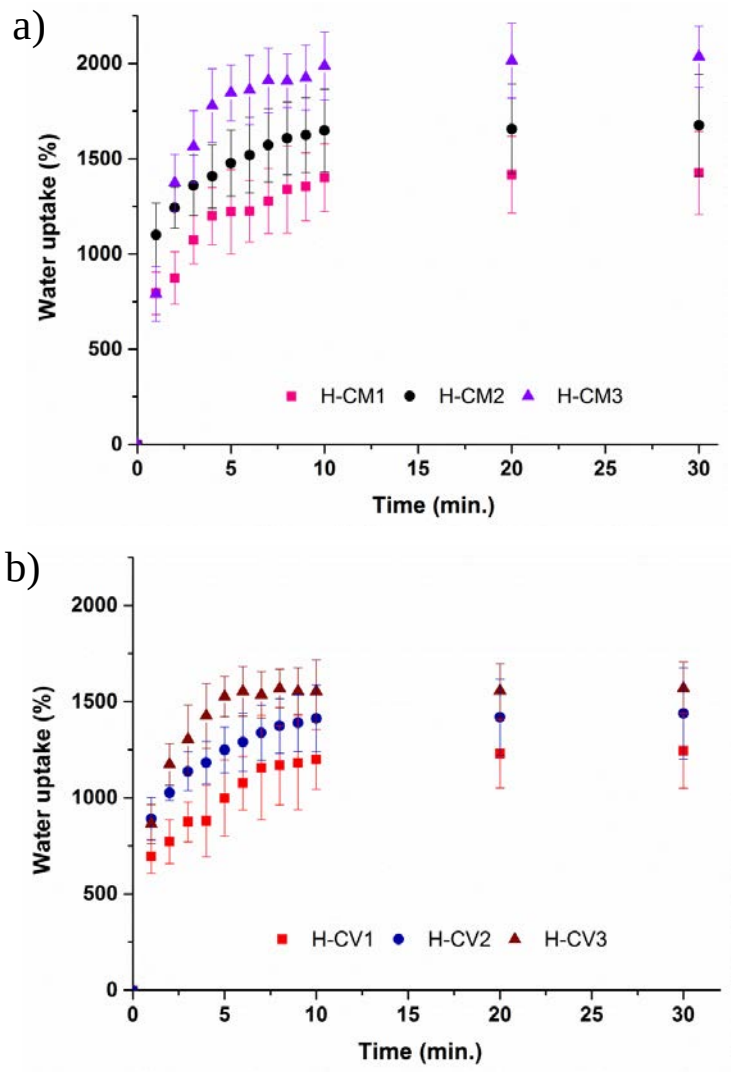


Figure 5.14. Water uptakes of hydrogels in water. a) Hydrogels prepared by bismaleimide-functionalized RAFT polymers b) Hydrogels prepared by bisvinyl-functionalized RAFT polymers (All data are in triplicate).

The viscoelastic gel properties of water-swollen hydrogels were examined via dynamic frequency scan analysis. The measurements revealed that the storage and loss moduli of networks show low oscillation frequency dependency indicating the viscoelastic gel behavior (Figure 5.15). The storage modulus (G′) and loss modulus (G″) values were ranging from 10^1 to 10^4 Pa and for the all samples tested, the storage modulus was found to be higher than the loss modulus which is a distinguishing rheological property of elastic solid materials. It was observed that the feed of DEGMA in hydrogel composition affects the rheological properties as stronger viscoelastic system was obtained with increasing DEGMA content.

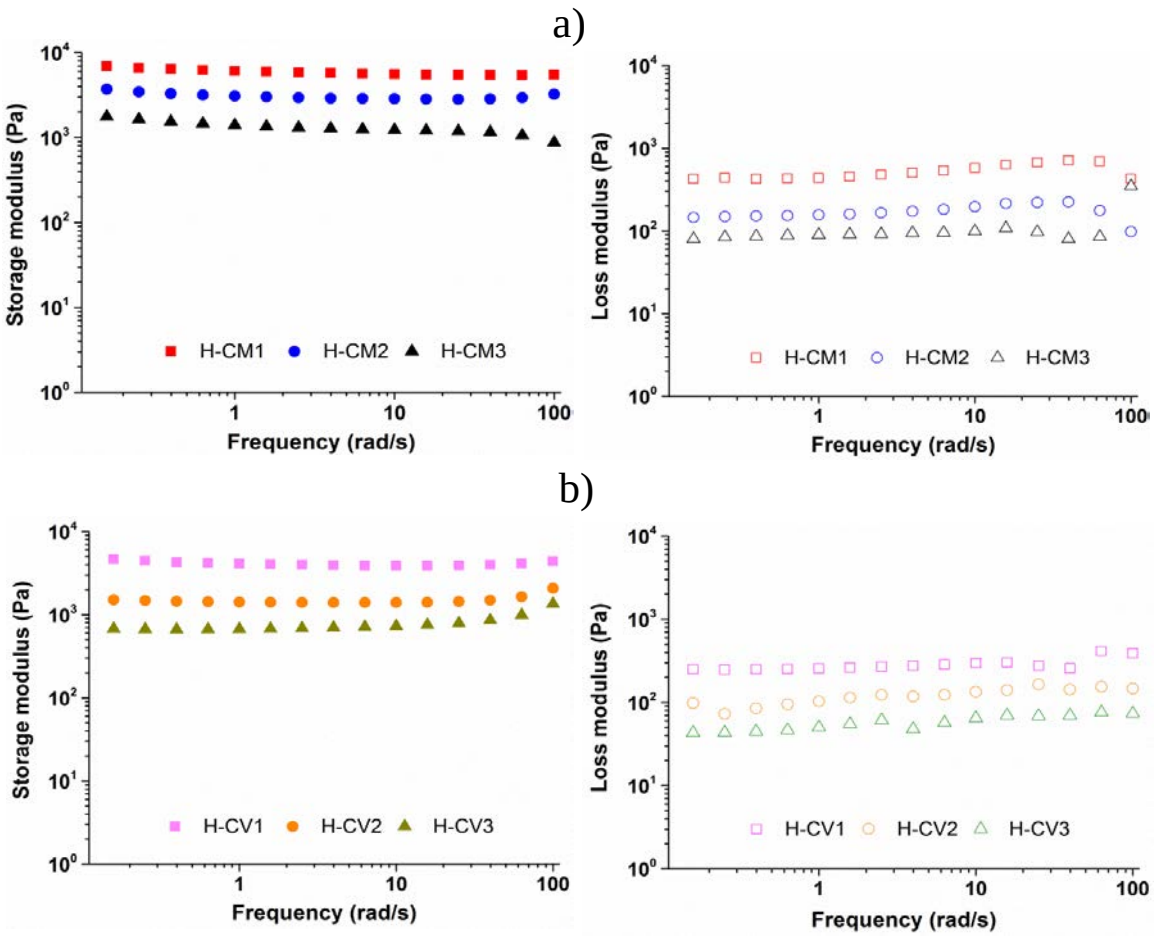


Figure 5.15. The frequency dependence of storage and loss moduli of hydrogels a) prepared by bismaleimide-functionalized RAFT polymers b) prepared by bisvinyl-functionalize RAFT polymers.

5.3.4. Drug Loading and Release Studies

Loading drugs to hydrogels and governing the controlled release of them to external media is an important task in bio-related applications of hydrogels. Most of the strategies to load the drugs into hydrogels are based on the encapsulation of the drug molecules into the network that can be maintained by in-situ loading or post-loading. In in-situ loading, drugs or drug-polymer conjugates are mixed with polymer precursors during gel formation to accomplish drug encapsulation simultaneously. In contrast to this strategy, in post-loading method, hydrogel matrix is first formed and the drug is absorbed to the network. For this absorption method, diffusion is the main force for drug uptake. The release of the drug molecules from hydrogel is still largely driven by the diffusion in which drug molecules drive from inner gel matrix to outer media through the porous structure. For

both drug loading and release, diffusion of drug molecules to or from hydrogel matrix is affected by important parameters such as, crosslinking density, structure of hydrophilic polymer backbone, swelling capacity of gel and additional molecular interactions between drug molecules and gel matrix. In cyclodextrin containing hydrogels, the inclusion complex formation of cyclodextrin with hydrophobic drug molecules offer another mean of drug loading and release providing an example of affinity based-systems.

The drug loading and release properties of hydrogels were studied using a poorly water soluble model drug puerarin, a traditional Chinese medicine used to treat glaucoma (Figure 5.16). Hydrogels prepared from HEMA/DEGMA-based polymers and β-CD$(SH)_7$ were loaded by puerarin using solution absorption method. Hydrogel disks swollen in water were soaked in 0.80 mg/mL drug solution and the drug loading was monitored spectrophotometrically until equilibrium was reached after 24 h incubation. The total absorbed drug amounts determined from the initial and final concentrations of soaking solutions were in between 1.50 % and 2.70 % (Table 5.2). Hydrogel drug loading capacities were found to be affected by the type of hydrophilic monomer. For both types of hydrogels obtained by nucleophilic and radical thiol-ene reactions, increasing the HEMA content caused an increase in total loaded drug. This result was attributed to the higher hydrophilic character of HEMA based polymer making a positive contribution to the amount of drug diffused in aqueous phase.

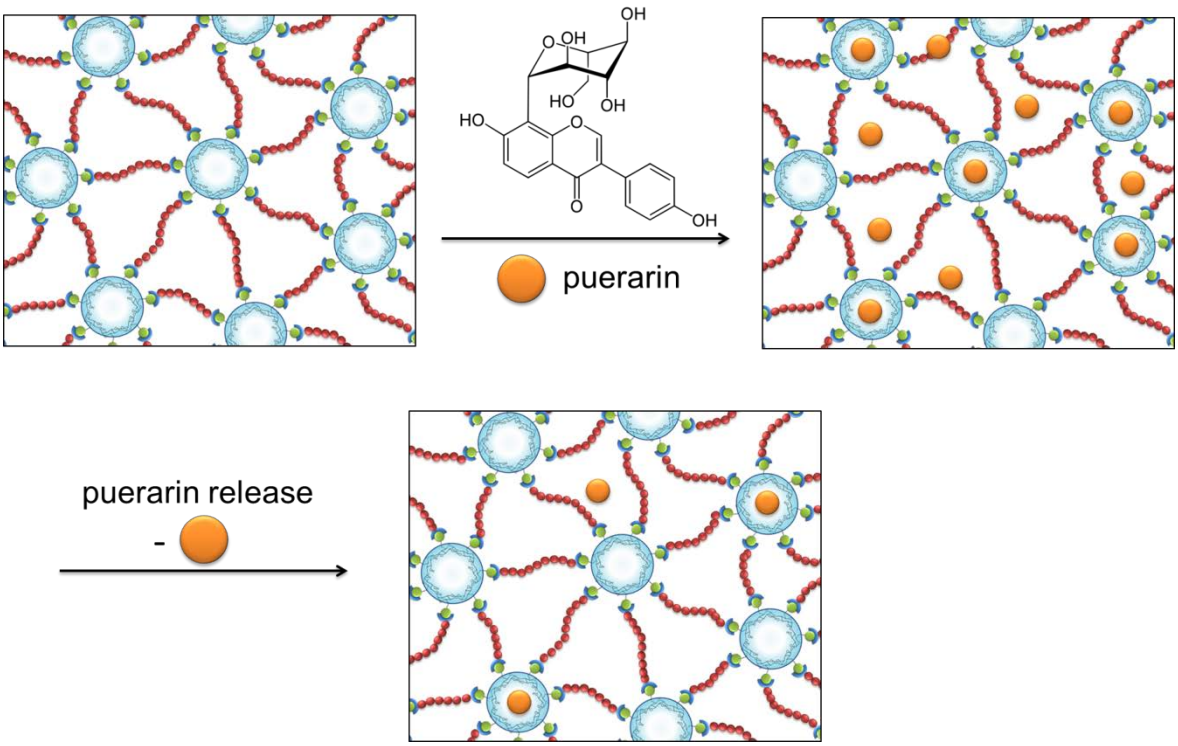


Figure 5.16. Schematic illustration of drug loading to cyclodextrin-containing hydrogels.

For the release studies, hydrogels loaded with puerarin were gently washed with distilled water and placed in a 15 mL release medium. In order to mimic sink conditions, a 5 mL release medium was replaced with a fresh solution with regular time intervals. The drug release was monitored by collecting the release samples and analyzing via UV spectrophotometer. As shown in Figure 5.17, all hydrogels exhibited an initial burst release which could be ascribed to the removal of free drug in aqueous phase and adsorbed drug on backbone of the hydrogel. The amount of burst released drug is increasing with the increase in HEMA content of both nucleophilic and radical thiol-ene hydrogels. In both systems, DEGMA based hydrogels showed lower initial burst and more sustained release.

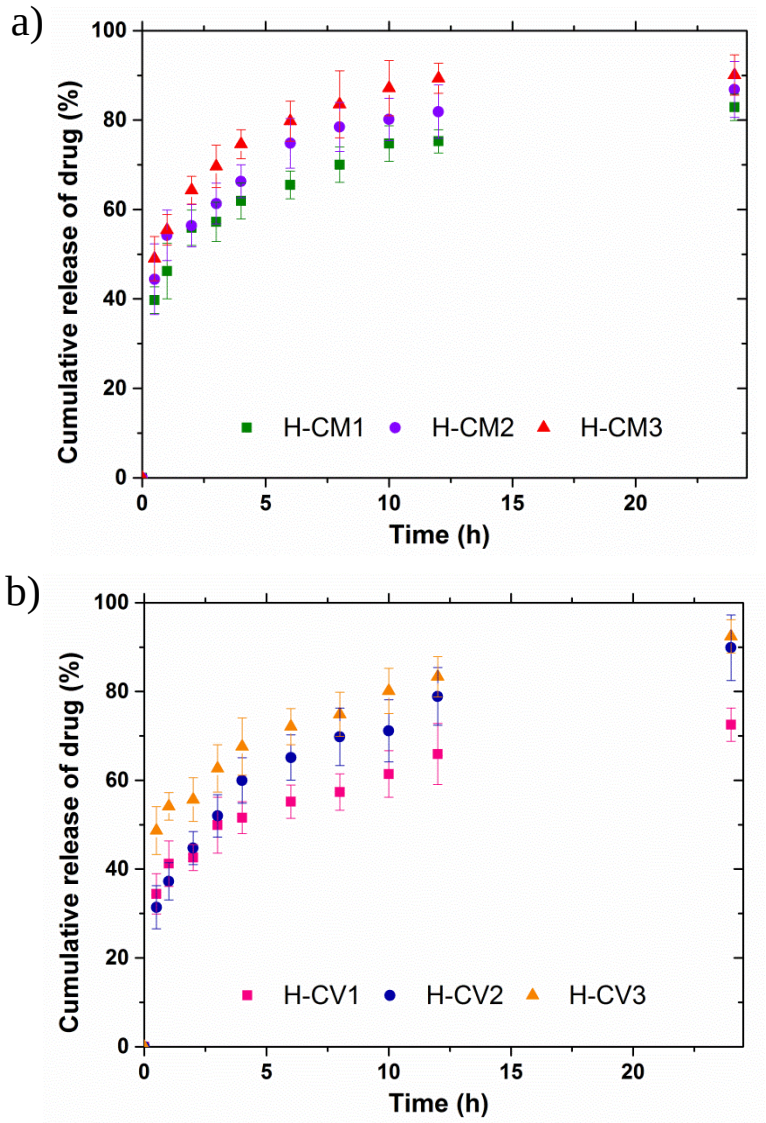


Figure 5.17. Cumulative release of puerarin from hydrogels a) H-CM(1-3) and b) H-CV(1-3).

5.4. Conclusions

In this project, utilization of RAFT-mediated telechelic polymers in post-polymerization functionalization reactions and fabrication of β-cyclodextrin-containing hydrogels was demonstrated. 2-hydroxyethyl methacrylate (HEMA) and/or di(ethylene glycol) methyl ether methacrylate (DEGMA)-based hydrophilic polymers were synthesized using reversible addition-fragmentation chain transfer (RAFT) polymerization. Thiol reactive maleimide and vinyl functional groups were placed at polymer end groups by using functional group containing CTAs and post-polymerization modification of

existing polymers. The reactive polymers were shown to be efficiently functionalized with thiol containing model compounds. The end group functional polymers were also utilized in hydrogel fabrication. Obtained hydrogels comprising different RAFT polymers were characterized in terms of physical properties such as water uptake capacity, surface morphology and rheological behaviors. These β-cyclodextrin containing hydrogels have been tested on drug sorption and controlled release by employing puerarin as a model drug molecule. The methodology of synthesizing telechelic RAFT polymers depicted here is believed to find potential application in polymer modification and design of hydrogel based controlled drug release systems.

6. CLICKABLE POLY(ETHYLENE GLYCOL)-BASED COPOLYMERS USING AZIDE-ALKYNE CLICK CYCLOADDITION MEDIATED STEP-GROWTH POLYMERIZATION

The materials in this chapter have been adapted with modifications from the following article: Arslan M., O. Gok, R. Sanyal, A. Sanyal, *Macromolecular Chemistry and Physics*, Vol. 215, pp. 2237-2247.

6.1. Introduction

Design and synthesis of reactive polymers that allow incorporation of desired molecules at post-polymerization stage have gained increased attention recently [139–141]. Polymeric scaffolds with precisely decorated functionalities have found applications in polymer based drug delivery [142, 143], biomolecular immobilization [146], fabrication of polymeric coatings [147] and organic-inorganic hybrids [148]. In many applications, the attachment of target molecules on polymers requires fast and clean procedures. For this reason, much of the effort has been dedicated to adapt the efficient chemical transformations towards polymer functionalization and post-polymerization modification has emerged as a powerful tool for fabrication of functional materials.

Post-polymerization modification or post-polymerization functionalization refers to the chemical modification of polymers with target molecules after the polymerization stage. Usually, desired post-functionalizable groups are placed on the polymer chain by either choosing reactive group containing initiators or by incorporating post-functionalizable monomers during polymerization (Figure 6.1). In applications that necessitate the presence of multi-functional groups on polymer chain, copolymerization of reactive monomers with other monomers give the opportunity of installing reactive functionalities along the backbone as side units.

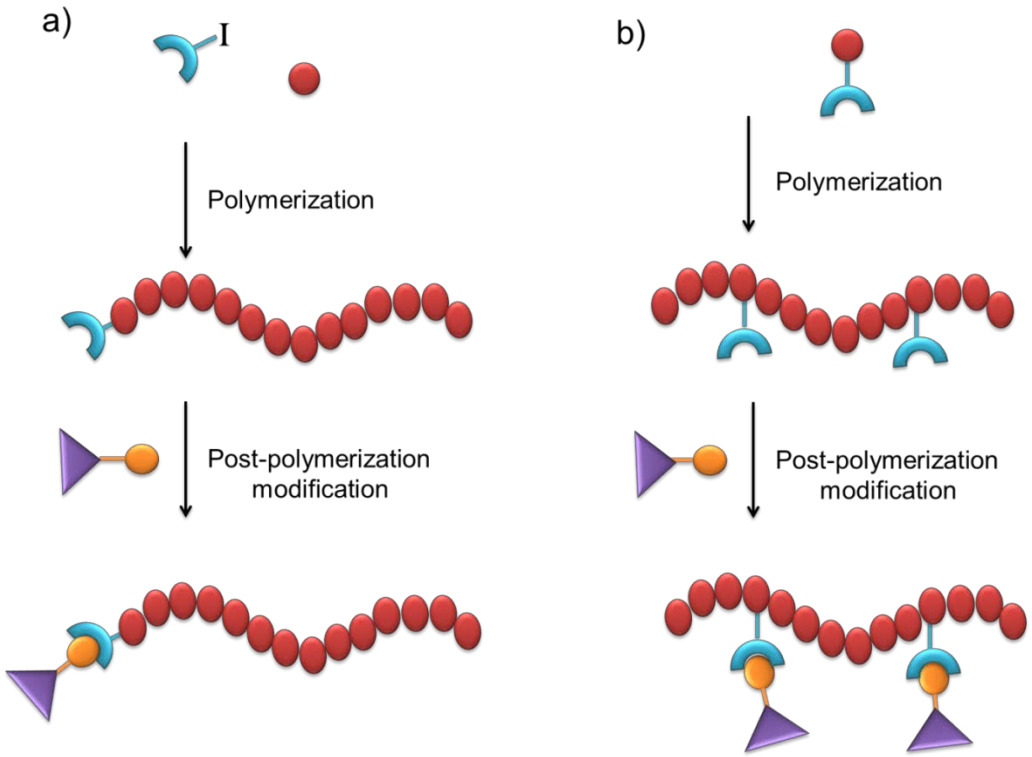


Figure 6.1. Synthesis and post-polymerization modification of polymers carrying functionalizable groups a) at initiator and b) at monomers.

The method of obtaining functionalizable groups at side chains provides tuning of side chain functional group concentration and allows the installment of desired molecules in multiple copies to the polymer backbone. Various functional groups including alkenes, activated esters, alkynes and azides, pyridiyl disulfides, activated dienes and dienophiles have been incorporated into polymers as pendant groups and these reactive groups have been shown to efficiently functionalized by adapting different chemical transformations [147–150]. Much of these transformations rely on ‘click’ chemistry based methodologies. Beside the advancements in post-polymerization modification of polymer obtained via chain growth polymerization, there is also growing research interest in functionalization of polymers obtained via step growth polymerization [153]. Efficient chemical transformations based on click strategies serve not only in synthesis of polymers via step growth fashion, but they also provide useful toolbox in post-polymerization functionalization of polymers in demanded applications.

[3+2] Huisgen type 1,3-dipolar cycloaddition is a cycloaddition reaction between an azide and an alkyne to give a 1,2,3-triazole. The reaction is usually implemented by using a copper catalyst yielding a 1,4-disubstituted triazole (Figure 6.2). Since the first attempts of developing a new polymerization technique based on copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of an azide and an alkyne [152, 153], click polymerization has emerged as an attracting concept in preparation of polymers via step-growth manner. The strategy is based on the coupling of homobifunctional alkyne and azide monomers or α-azide-ω-alkyne heterobifunctional polymerizable units in a polyaddition fashion. Due to the orthogonal, selective and highly efficient nature of click reaction, this polymerization technique remains as powerful method for polymer synthesis. Diverse polymeric structures have been prepared via utilization of the copper(I)-catalyzed azide-alkyne click reaction [154–156]. However, in the context of post-polymerization functionalization, there are limited examples of click polymers that allow post-polymerization modification.

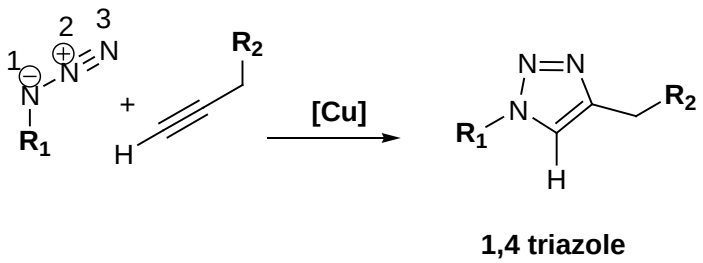


Figure 6.2. Copper catalyzed [3+2] Huisgen type 1,3-dipolar cycloaddition reaction.

As depicted in the previous sections, the utilization of Michael type addition of thiols on maleimides has been extensively exploited in polymer functionalization. Due to high reaction yields, high selectivity and catalyst free reaction conditions, this versatile chemistry have provided an important tool for engineering materials. Especially in designing polymeric scaffolds for biomolecular immobilization of relevant molecules (enzymes, proteins, peptides), it is important to achieve precisely appointed and well defined systems for quantitative assessment of such conjugates. For this purpose, reactive cysteine residues of proteins provide an opportunity due to the presence of thiol groups. Effective conjugation of cysteine thiols to maleimide groups of polymers provides site specific functionalization of polymeric materials in a fast and tolerant manner. Maleimide

is also an excellent dienophile and gives another important click reaction, i.e. Diels-Alder reaction with electron rich dienes. Diels–Alder chemistry of maleimides with various dienes has also been exploited in polymer synthesis and functionalization, demonstrating the versatility of maleimide based systems in engineering polymeric materials [157–161].

In this project, design, synthesis and post-polymerization functionalization of linear PEG-based polytriazole copolymers containing thiol-reactive maleimide functional groups as pendant side chains is described (Figure 6.3) [164]. Copper(I)-catalyzed Huisgen-type 1,3-dipolar cycloaddition reaction was utilized to prepare copolymers through combination of diazide and dialkyne functionalized triethylene glycol derivatives along with a dialkyne-functionalized furan-protected maleimide-containing monomer. After the polymerization, microwave assisted retro-Diels-Alder reaction was utilized to unmask the pendant maleimide groups by removal of the furan moieties. Post-polymerization modifications of these PEG-based copolymers containing the furan-protected maleimide group was accomplished using the photo-initiated radical thiol-ene reaction, whereas polymers containing reactive maleimide group were functionalized using the Diels-Alder and the nucleophilic thiol-ene reaction. The nucleophilic thiol-ene reaction was utilized to fabricate hydrogels by treatment of the maleimide containing PEG-polymers with dithiol-containing cross-linkers. Facile functionalization of thus obtained hydrogels under mild conditions was demonstrated through treatment of the residual maleimide groups within the hydrogel with a thiol-containing fluorescent dye.

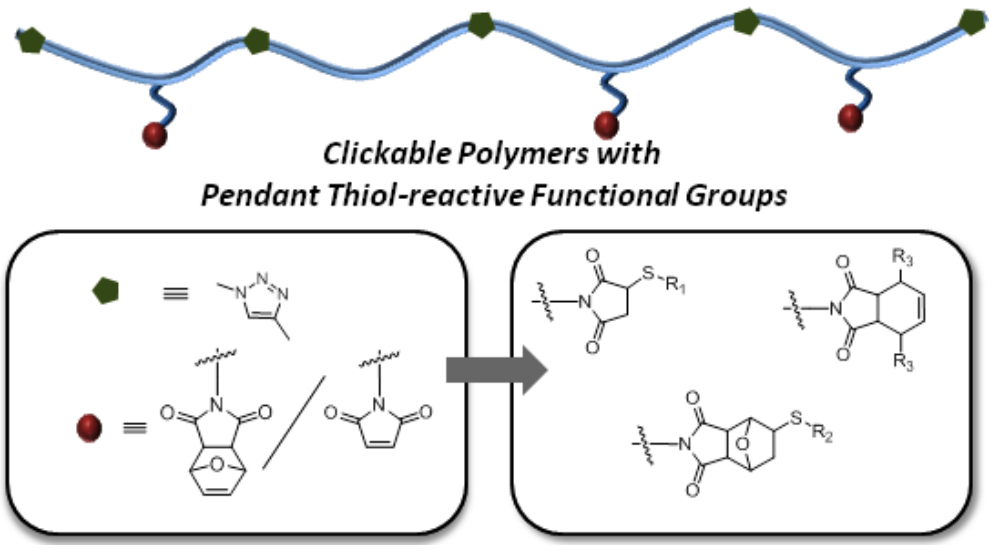


Figure 6.3. Polytriazole polymers with side chain functionalizable groups.

6.2. Experimental

6.2.1. Materials and Characterization

The reagents and solvents were purchased from commercial sources and used as received unless otherwise noted. Triethylene glycol and sodium azide were purchased from Merck. Sodium hydride, propargyl bromide, Cu(I) bromide, Cu(I) iodide, bromotris(triphenylphosphine)copper(I), N,N,N′,N′,N″-pentamethyldiethylenetriamine (PMDTA), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), N,N-Diisopropylethylamine (DIPEA), benzyl mercaptan, 2,2-dimethoxy-2-phenylacetophenone (DMPA), 3,6-di-2-pyridyl-1,2,4,5-tetrazine, anthracene and 2,2′-(Ethylenedioxy)diethanethiol were obtained from Aldrich. Homobifunctional triethylene glycol monomers with alkyne (diyne-TEG) [165] and azide (diazide-TEG) [166] groups at their termini were synthesized according to reported procedures and confirmed with spectroscopic analysis. Compound diyne-FuMAL was synthesized according to previously published procedure [167].

The gel permeation chromatography (GPC) measurements of polymers were carried out using a Shimadzu GPC analysis system with PSS WinGPC Unity software. PSS Gram (length/ID 300 mm × 8 mm, 10 µm particle size) column was calibrated with polymethyl methacrylate standards, using refractive index detector (RID-10A). DMAc was used as eluent at a flow rate of 1mL/min at 30 ^{o}C. NMR spectra were recorded on a Varian 400-MHz spectrometer. The microwave assisted reactions were performed using a Biotage® Initiator synthesizer. Reactions were carried in single-use 10 mL reaction vessels and septa designed for high temperature/pressure reactions were used. Thermogravimetric analysis (TGA) was carried out on a TA Instruments at a heating rate of 10 ^{o}C/min under nitrogen atmosphere.

6.2.2. General Procedure for the Polymerization of Diyne-TEG and Diazide-TEG Monomers using Different Copper Catalysts

Diyne-TEG (226.20 mg, 0.1 mmol), diazide-TEG (200.20 mg, 0.1 mmol) and copper catalyst/ligand mixture (CuBr/PMDTA : 0.1 mmol/0.2 mmol; CuI/DBU : 0.1 mmol/1.0 mmol; $Cu(PPh_3)_3$/DIPEA : 0.1 mmol/1.0 mmol) was dissolved in DMF (4 mL) in a sealed tube equipped with a magnetic stir bar. The mixture was purged with nitrogen for 15 minutes and allowed to stir at 25 ^{o}C for 12 h. After polymerization, the catalyst was

removed by passing the mixture through neutral aluminum oxide. The solvent was removed under reduced pressure and remaining crude polymer was re-dissolved in minimum amount of dichloromethane before precipitating into cold THF. After drying in vacuum polymers were obtained as waxy liquids. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.69 (s, 2H, $OCH_2C{=}CH$), 4.63 (s, 4H, $NCCH_2O$), 4.48 (bt, 4H, NCH_2CH_2O), 3.79-3.52 (backbone CH_2's, 20 H, stack).

6.2.3. General Procedure for the Polymerization of Diyne-FuMAL, Diyne-TEG and Diazide-TEG Monomers with CuBr Catalyst (Pt-FuMAL)

Typical polymerization procedure for Pt-FuMAL-1: Diyne-FuMAL (49.95 mg, 0.1 mmol), diyne-TEG (203.60 mg, 0.9 mmol), diazide-TEG (200.20 mg, 1.0 mmol), CuBr (14.30 mg, 0.1 mmol) and PMDTA (41.71 µL, 0.2 mmol) was dissolved in DMF (4 mL) in a sealed tube equipped with a magnetic stir bar. After 15 minutes nitrogen purge of the solution, reaction mixture was stirred at 25 ^{o}C for 12 h. The catalyst was removed by passing the mixture through neutral aluminum oxide. The solvent was removed under reduced pressure and remaining crude polymer was re-dissolved in minimum amount of dichloromethane before precipitating into cold THF. The polymer was obtained as waxy liquid (67 %). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) 7.67 (s, $OCH_2C{=}CH$, 2H), 7.45 (s, $CH_2CH_2C{=}CH$, 2H), 6.47 (s, $CH{=}CH$, 2H), 5.20 (s, CH bridgehead protons, 2H), 4.63 (s, $NCCH_2O$, 4H), 4.47 (t, NCH_2CH_2O, 4H), 4.26-4.15 (m, OCH_2C ester, 4H), 4.06-3.95 (m, OCH_2CH_2 ester, 2H), 3.79-3.51 (backbone CH_2's, stack), 2.97 (t, $NCCH_2$, 4H), 2.83 (s, bridge protons, 2H), 2.71 (t, $NCCH_2CH_2$, 4H), 1.87 (m, $NCCH_2CH_2$, 2H), 1.23-1.19 (two singlets, CH_3, 3H).

6.2.4. General Procedure for Post-polymerization Activation of Maleimide Functional Groups (Pt-MAL)

Polymer Pt-FuMAL-1 (50.0 mg) was dissolved in 1:1 mixture of chloroform/acetonitrile (2 mL) and irradiated in microwave for 60 min. (110 ^{o}C). On cooling at room temperature, solvent was evaporated in vacuo to yield polymer Pt-Mal-1 in quantitative yield. The obtained polymer was characterized using ^{1}H NMR spectroscopy.

6.2.5. Post-polymerization Functionalization of Pt-MAL-1 Polymer *via* Nucleophilic-Thiol-Ene Reaction

Polymer Pt-MAL-1 (50.0 mg, 0.004 mmol) and benzyl mercaptan (6.20 mg, 0.05 mmol) were dissolved in 2 mL of dichloromethane and triethylamine (1.0 μL, 0.007 mmol) was added to the solution. The mixture was stirred at room temperature for 12 h. After reaction, the mixture was precipitated in cold THF affording the thiol appended polymer.

6.2.6. Post-polymerization Functionalization of Pt-MAL-1 Polymer via Diels-Alder Cycloaddition Reaction

A solution of polymer Pt-MAL-1 (50.0 mg, 0.004 mmol) and anthracene (2.30 mg, 0.013 mmol) in DMF (1 mL) was heated at 120 ^{o}C for 24 h in the dark. The reaction mixture was concentrated in vacuo to remove the solvent and the crude product was dissolved in minimum amount of dichloromethane before precipitating in cold THF.

6.2.7. Functionalization of Pt-FuMAL-1 Polymer via Radical-Thiol-Ene Reaction

Polymer Pt-FuMAL-1 (50.0 mg, 0.004 mmol), benzyl mercaptan (6.20 mg, 0.05 mmol) and DMPA (1.80 mg, 0.007 mmol) were dissolved in the 200 μL of DMF. The mixture was purged with nitrogen for 10 min. and then was exposed to UV light at 365 nm for one hour. After reaction, the resulting polymer was purified by precipitating in cold THF.

6.2.8. Hydrogel Synthesis via Crosslinking of Maleimide-containing Polymer

The polymer Pt-MAL-1 (50.0 mg, 0.004 mmol) was placed in a vial and dissolved in dichloromethane (200 μL). A solution of 2,2′-(ethylenedioxy)diethanethiol (1.64 mg, 0.009 mmol) and triethylamine (0.10 μL, 9 x10^{-4} mmol) in dichloromethane (100 μL) was then added to this solution. The mixture was briefly sonicated to assist homogenous gelation. After hydrogel formation, unreacted reagents were removed by washing the gel with THF followed by distilled water several times. The swollen hydrogel sample was frozen and lyophilized to yield dried hydrogel.

6.2.9. Functionalization of Hydrogel with Thiol-containing Dye

Hydrogel containing free maleimide groups was functionalized via SAMSA fluorescein thiol conjugation. First, protecting group of the dye was removed by following the procedure indicated by the supplier. Briefly, 10 mg of SAMSA fluorescein was dissolved in 0.1 M NaOH and incubated at room temperature for 15 minutes to remove the acetyl protecting group. Then, the solution was neutralized with concentrated HCl and buffered to pH 7 with 0.5 M sodium phosphate. The amount of free maleimide groups in a 10 mg hydrogel sample was calculated and a ten-fold excess of activated SAMSA fluorescein thiol solution was added. After incubation at room temperature for 12 h, hydrogel was washed several times with deionized water before analysis of fluorescence microscopy. As a control experiment, a sample of hydrogel was incubated with acetyl protected SAMSA fluorescein thiol dye.

6.3. Results and Discussion

6.3.1. Synthesis of Maleimide Side Chain Functionalized Click Step Growth Polymers

Cu-catalyzed azide-alkyne cycloaddition polymers comprising reactive side-chain groups were synthesized via utilizing click polyaddition of homobifunctional monomers containing complementary click coupling functionalities. A diyne functionalized furan protected maleimide containing monomer (diyne-MAL) was copolymerized with hydrophilic diyne (diyne-TEG) and diazide (diazide-TEG) triethylene glycol derivatives. The strategy involves a A_1-A_1/B_1-B_1/B_2-B_2 type combination of diazide and dialkyne containing monomers and the amount of dialkyne monomer containing reactive maleimide functionality was tailored to achieve desired degree of functionalization.

As a first study, the polymerizability of diyne-TEG and diazide-TEG monomers was investigated using different copper catalyst systems (Figure 6.4). The polytriazoles with hydrophilic oligo(ethylene glycol) backbones were synthesized using CuBr, CuI and $Cu(PPh_3)_3$ catalyst at room temperature. Dimethylformamide (DMF) was chosen as the reaction solvent due to its high solvating ability towards the hydrophilic diyne-TEG,

diazide-TEG monomers and hydrophobic diyne-MAL monomer as well as the obtained polytriazoles. The polymerization results were summarized in Table 6.1 (Items 1-3).

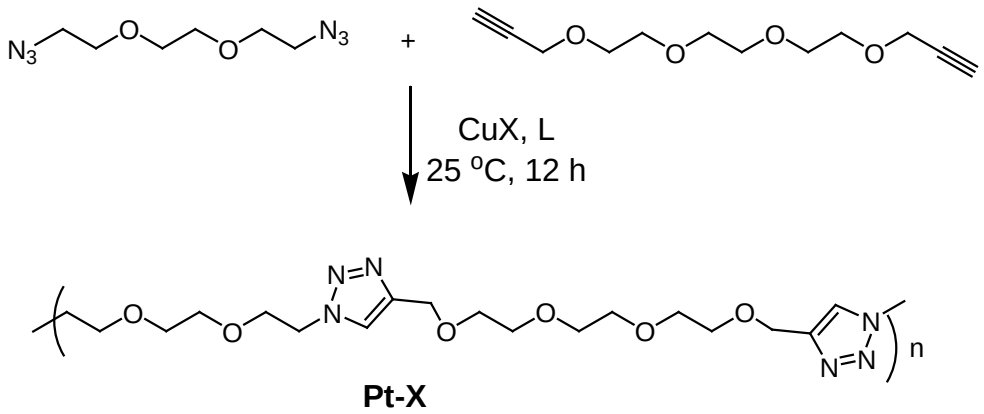


Figure 6.4. Syntheses of polytriazoles from diyne-TEG and diazide-TEG monomers with different copper catalysts (CuX: CuBr, CuI or $Cu(PPh_3)_3$).

Table 6.1. Properties of copolymers obtained by Cu-catalyzed polyaddition of diyne and diazide monomers.

Item	**Polymer**	**Catalyst**	**$[M]_1$:$[M]_2$:$[M]_3$[a]**	**F[b] (%)**	**$M_{n,SEC}$[c] (g/mol)**	**M_w/M_n[c] (g/mol)**	**Conv. (%)**
1	Pt-1	CuBr/PMDETA	1 : 1 : 0	-	14400	1.72	72.4
2	Pt-2	CuI/DBU	1 : 1 : 0	-	14200	2.23	66.3
3	Pt-3	$Cu(PPh_3)_3$/DIPEA	1 : 1 : 0	-	5600	1.94	51.7
4	Pt-FuMAL-1	CuBr/PMDETA	1 : 0.9 : 0.1	8.73	11700	1.60	66.7
5	Pt-FuMAL-2	CuBr/PMDETA	1 : 0.8 : 0.2	12.80	9400	1.68	58.9
6	Pt-FuMAL-3	CuBr/PMDETA	1 : 0.7 : 0.3	21.20	7700	1.76	69.2

[a] Mol equivalent; $[M]_1$: diazide-TEG, $[M]_2$: diyne-TEG, $[M]_3$: diyne-FuMAL. [b] $F_{..}$ $[M]_3$ / ($[M]_2$ + $[M]_3$) Determined by ^{1}H NMR analysis. [c] Estimated by SEC eluted with DMAc, using poly(methyl methacrylate) standards.

According to SEC analysis, copolymers prepared from diyne-TEG and diazide-TEG monomers using CuBr and CuI catalysts have molecular weights around 14 kDa. Polytriazole obtained by CuBr catalyst showed narrower polydispersity index compared to CuI catalyst. A higher polymer conversion was also attained in case of CuBr/PMDTA system. The polymerization with $Cu(PPh_3)_3$ catalyst yielded relatively lower molecular weight copolymer. The lower performance of this catalyst was attributed to the side

reaction between (PPh_3) groups from the copper catalyst and azide functionalities (Staudinger reaction) which shows a detractive effect on polymerization [168]. The obtained polymers are practically soluble in common solvents like dichloromethane, chloroform, dimethylformamide and acetonitrile; however, they are not soluble in THF and dioxane. Copolymers were characterized by ^1H NMR spectroscopy and an example spectrum of copolymer Pt-1 in $CDCl_3$ is seen in Figure 6.5. The specific signal belonging to triazole proton was observed at 7.69 ppm. The signals of oligo(ethylene glycol) backbone were also properly assigned confirming the generation of polymers through polytriazole formation.

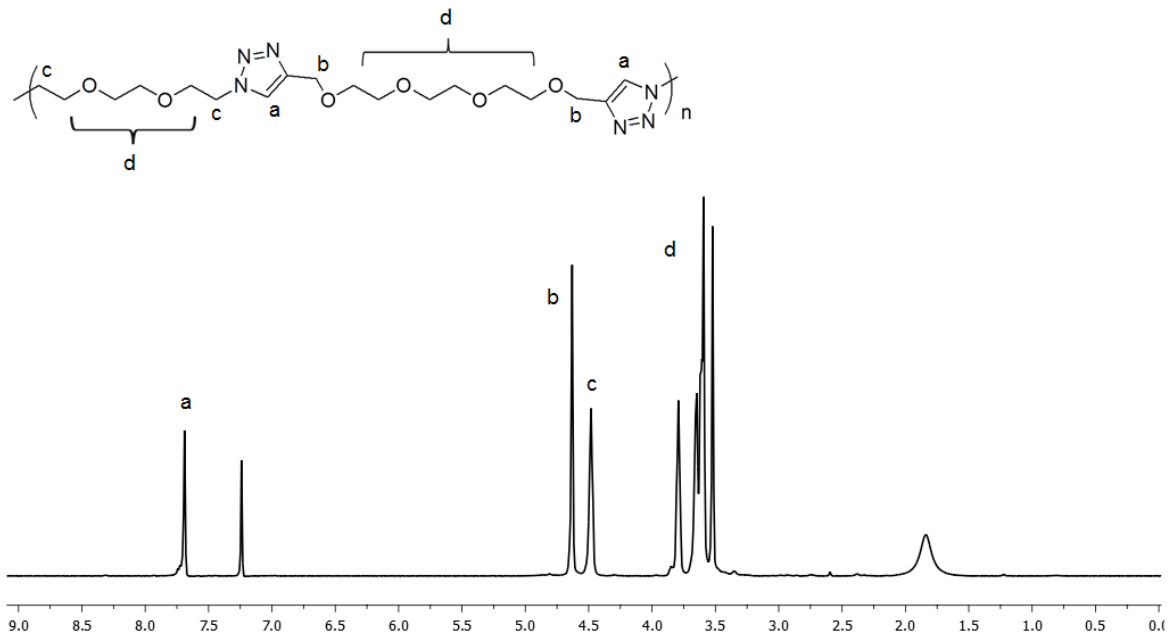


Figure 6.5. ^1H NMR spectrum of the polytriazole Pt-1 obtained by the polymerization of diazide-TEG and diyne-TEG monomers.

Diazide-TEG and diyne-TEG monomers were copolymerized with varying amounts of diyne-MAL monomer using CuBr/PMDTA catalyst system (Pt-FuMAL, Table 6.1, Items 4-6) (Figure 6.6). The polymerizations were carried out in DMF at room temperature. After polymerization, purification was carried out by precipitating polymers in THF to get rid of any unreacted monomers and low molecular weight fractions.

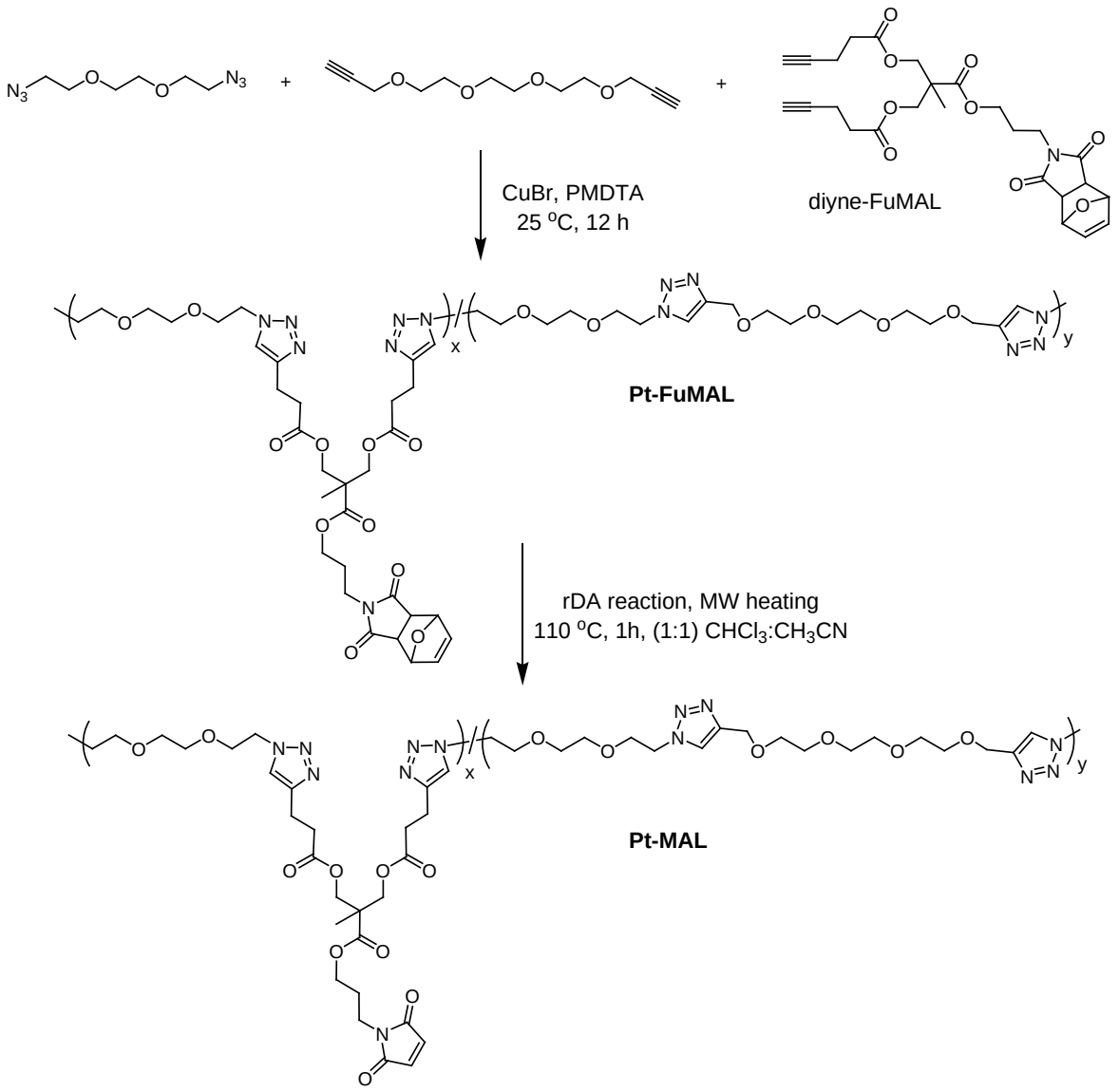


Figure 6.6. Synthesis of maleimide side chain functionalized PEG-based polytriazole copolymer.

The incorporation of diyne-MAL monomer to the copolymers was analyzed using ^{1}H NMR and successful incorporation was confirmed from the proton resonances around 6.47, 5.20 and 2.83 ppm belonging to the bicyclic moiety of the monomer (Figure 6.7). The newly formed triazole signal at 7.45 ppm and characteristic peaks coming from the ester arms of the monomer also evidences the monomer participation. The comparison of integration values of two triazole proton resonances has revealed the extent of incorporation of the diyne-MAL monomer to final copolymers. A lower incorporation of the monomer than aimed in the feed was observed. This could be explained by the steric bulk of the monomer as differs from the linear TEG derivatives. Another underlying reason

for low contribution might be the AA+BB type dimerized cyclization products that consumes the monomer to some extent, lowering the incorporation [169].

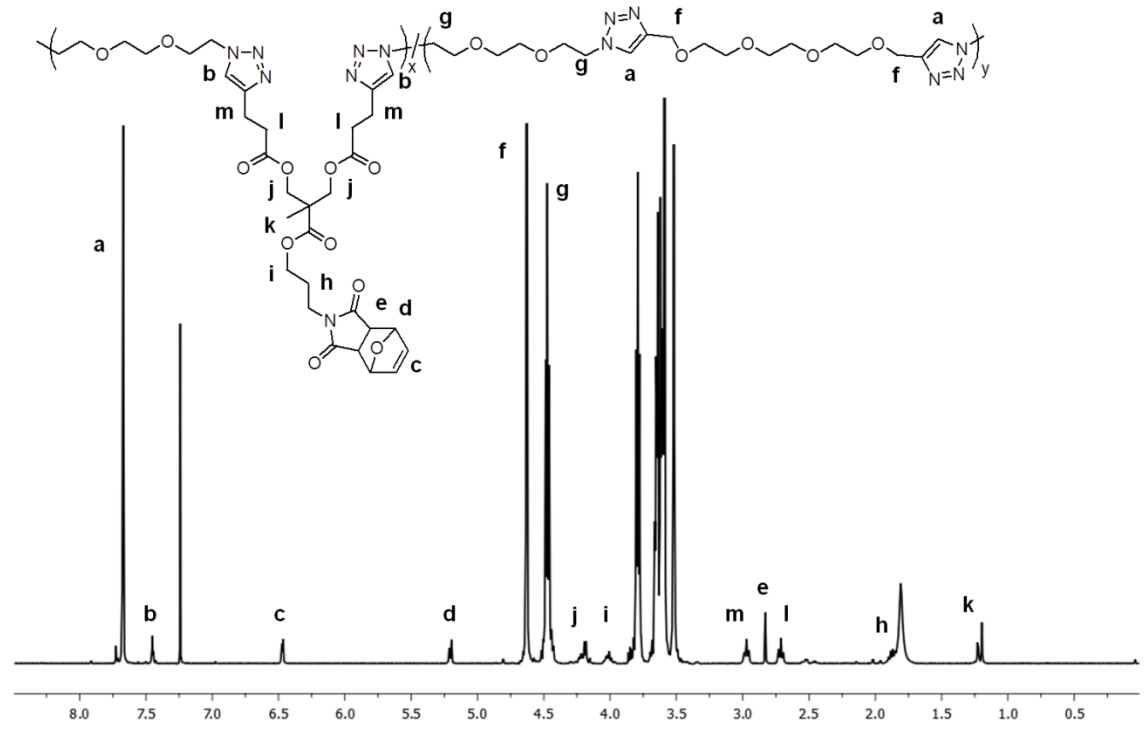


Figure 6.7. Representative ^{1}H NMR spectrum of copolymer Pt-FuMAL-1 containing furan-protected maleimide functional groups as side chains.

The masked maleimide groups at side chains were deprotected by utilization of retro-Diels-Alder reaction (Figure 6.6). First attempts to remove the furan protecting group in refluxing toluene or dioxane were unsuccessful due to poor solubility of polymers. The neat heating of polymers in vacuum also generated cross-linked materials. As an alternative strategy, the polymers were subjected to microwave heating at 110 ^{o}C in chloroform-acetonitrile mixture. In one hour complete removal of the furan protecting groups were achieved as revealed by the ^{1}H NMR analysis (Figure 6.8). The disappearance of proton resonances of bridgehead bicyclic structure and formation of a new peak at 6.68 ppm belonging to the maleimide double bond confirms the successful cycloreversion.

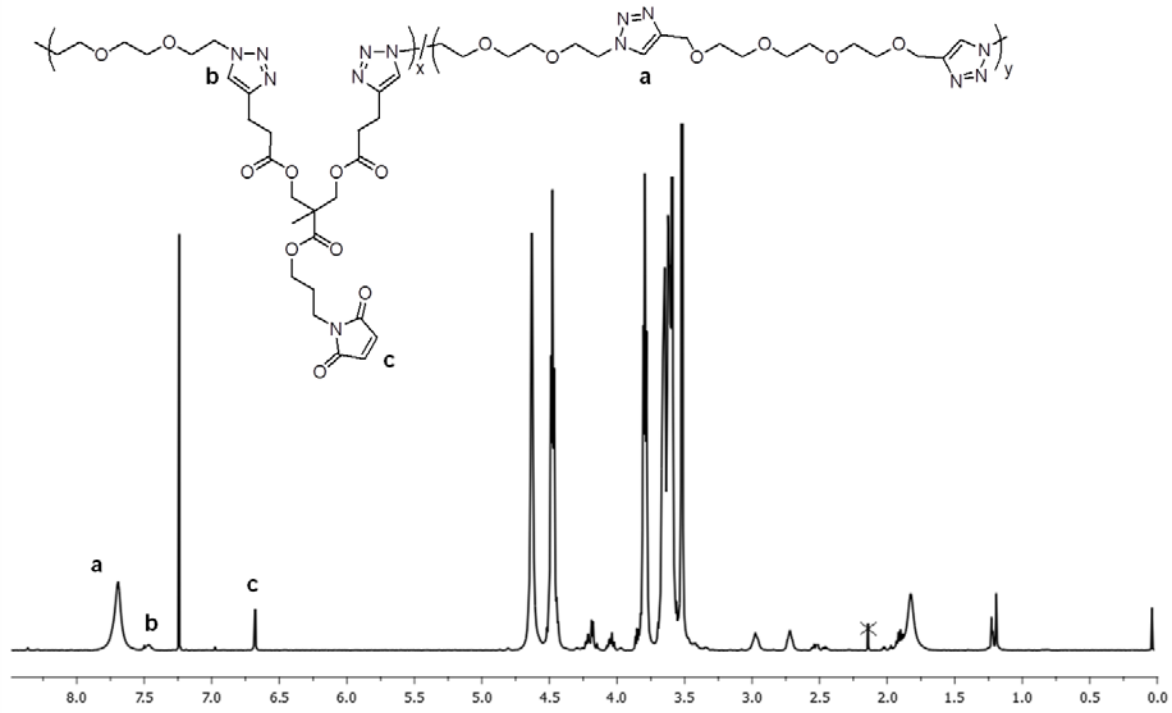


Figure 6.8. ^{1}H NMR spectrum of copolymer Pt-MAL-1 containing activated maleimide functional groups at side chains.

The weight loss due to the removal of furan moieties during rDA reaction was also determined by thermogravimetric analysis (TGA). The polymers showed an initial weight loss between temperatures of 90 to 150 ^{o}C during the loss of furan groups (Figure 6.9). An increasing weight loss is observed as the amount of monomer in polymer composition increased. According to SEC analysis no detrimental effect of rDA reaction on polymer structure was observed since the molecular weight and its distribution did not change significantly (Figure 6.10).

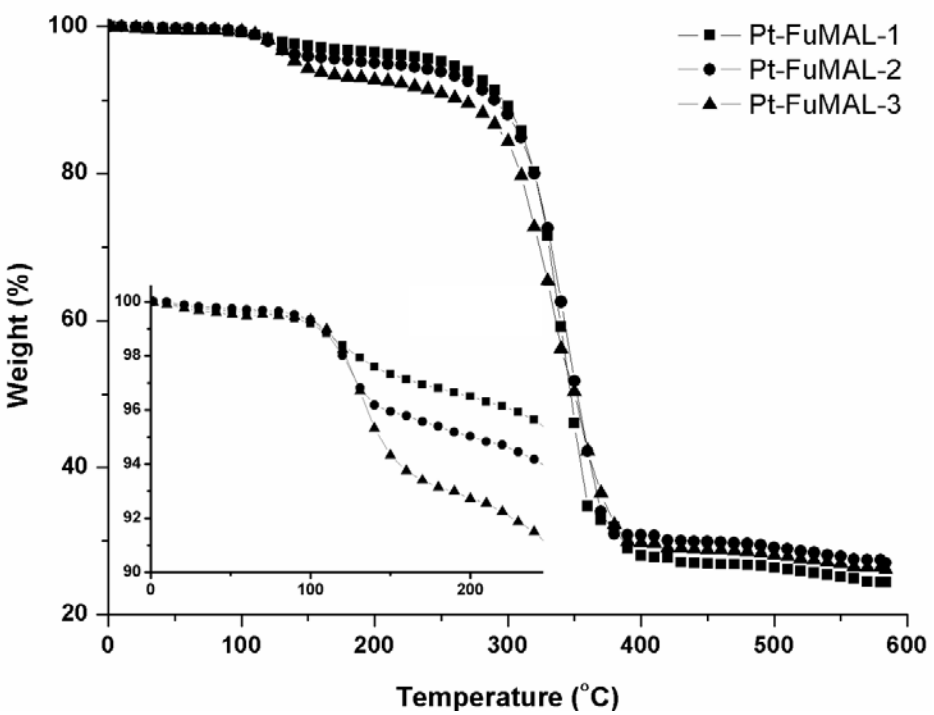


Figure 6.9. Thermogravimetric Analysis Plots of Copolymers Pt-FuMAL(1-3).

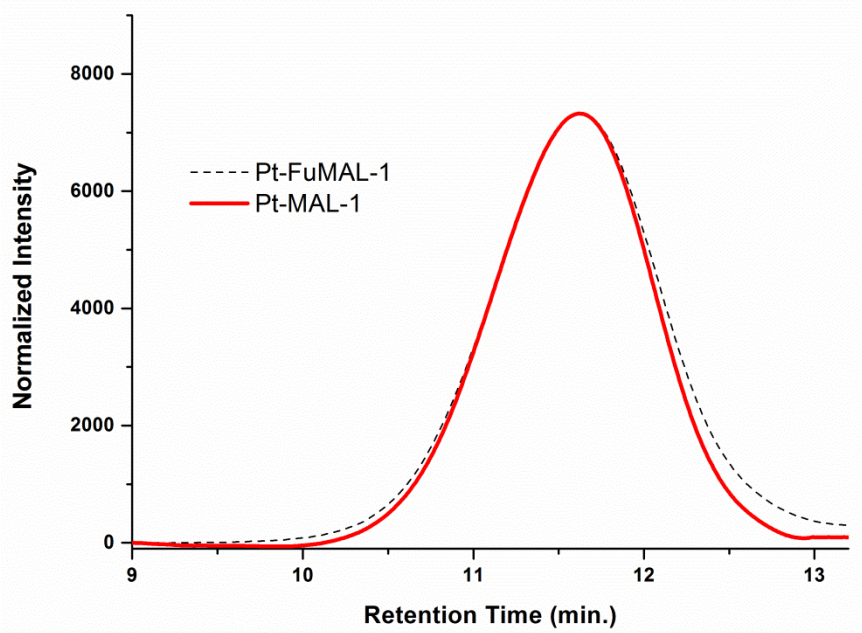


Figure 6.10. SEC traces of copolymer Pt-FuMAL-1, before and after retro-Diels-Alder reaction.

6.3.2. Functionalization of Polymers Containing Pendant Reactive Groups

Polymers carrying maleimide and furan-maleimide oxanorbornene groups as side chains along the copolymer backbone were functionalized with small model molecules by employing various click reactions. Functionalization of the maleimide groups was explored using the nucleophilic thiol-ene and the Diels-Alder cycloaddition reactions. As an alternative strategy for the conjugation of thiol-containing molecules to these polymers, the radical thiol-ene reaction was examined for the copolymers containing oxanorbornene moieties.

Post-polymerization functionalization efficiency of Pt-MAL-1 polymer with maleimide side chain functionalities was investigated by reacting it with benzyl mercaptan. It is well known that thiols are excellent reagents for site-specific attachment onto maleimide groups through a conjugate addition reaction. This Michael-type addition is generally catalyzed by a non-nucleophilic base. The amount of maleimide groups on polymer Pt-MAL-1 was calculated and 5 equivalent of benzyl mercaptan was used for complete conversion. The reaction was accelerated with catalytic amount of triethylamine (Figure 6.11). ^{1}H NMR spectroscopy indicated the successful attachment of molecule to the polymer from the disappearance of the proton resonance around 6.7 ppm and formation of new aromatic peaks coming from the thiol molecule. Additionally, proton resonances in the aromatic region arising due to the phenyl ring of benzyl mercaptan were also visible.

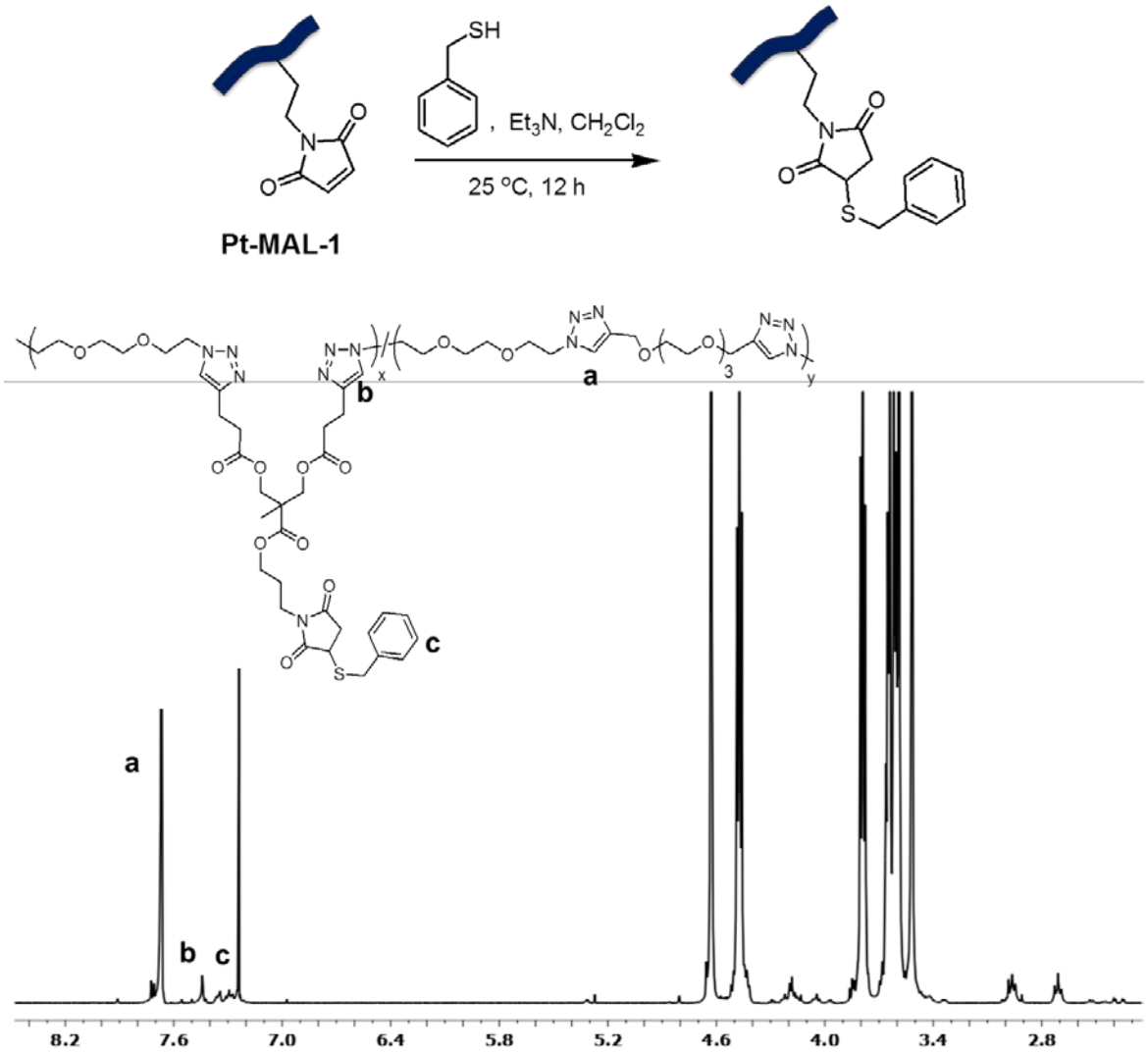


Figure 6.11. Synthetic transformation and ^{1}H NMR spectrum of nucleophilic thiol-ene functionalized copolymer Pt-MAL-1.

After confirming accessibility of maleimide groups for functionalization, another chemistry was employed for attachment of desired molecules. Beyond the susceptible conjugate addition reaction with thiols, maleimides are also excellent dienophiles for various dienes in Diels-Alder reactions. In order to study the post-polymerization functionalization of maleimide groups via Diels-Alder reaction, anthracene was chosen as a common diene. A slightly higher amount of anthracene from calculated maleimide ratio (1.3 eq) was reacted with polymer Pt-MAL-1 at 120 ^{o}C in DMF. The polymer functionalization was monitored by ^{1}H NMR after completing the reaction. It was found that, after 24 h reaction period, complete conversion was achieved as indicated by the loss of maleimide double bond peaks (Figure 6.12). Newly formed aromatic proton resonances

around 7.10-7.40 ppm also indicates the successful functionalization of maleimide groups with anthracene.

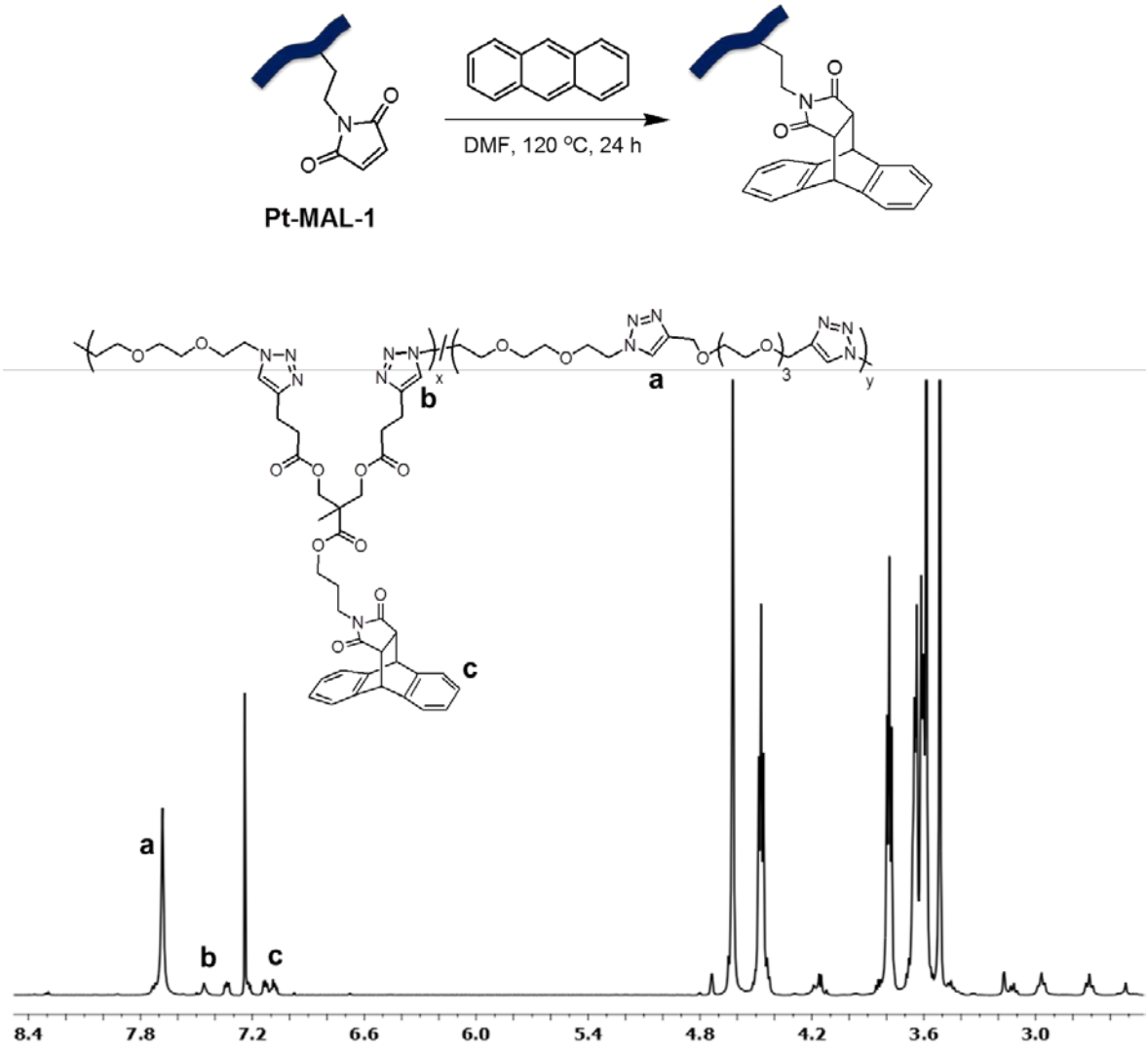


Figure 6.12. Synthetic transformation and ^{1}H NMR spectrum of Diels-Alder functionalized copolymer Pt-MAL-1.

Following the efficient post-polymerization modifications of maleimide side chain groups, the functionalization of polymer Pt-FuMAL-1 comprising furan/maleimide oxanorbornene scaffolds was also investigated using radical thiol-ene chemistry. Benzyl mercaptan was used as model compound for functionalization reaction. In order to increase the functionalization efficiency, five equivalent of benzyl mercaptan from calculated furan/maleimide cycloadduct ratio was used. After 1 h UV irradiation with the aid of 2,2-dimethoxy-2-phenylacetophenone (DMPA) photoinitiation, reaction was stopped and crude mixture was precipitated in cold THF to get rid of the excess reagent and residues.

Analysis of polymer with ^{1}H NMR showed the disappearance of the furan/maleimide cycloadduct double bond peak at 6.47 ppm as well as the signal coming from the bridgehead protons at 5.20 ppm (Figure 6.13). Furthermore, formation of new peaks due to the aromatic protons of benzyl mercaptan implied the successful radical thiol-ene functionalization.

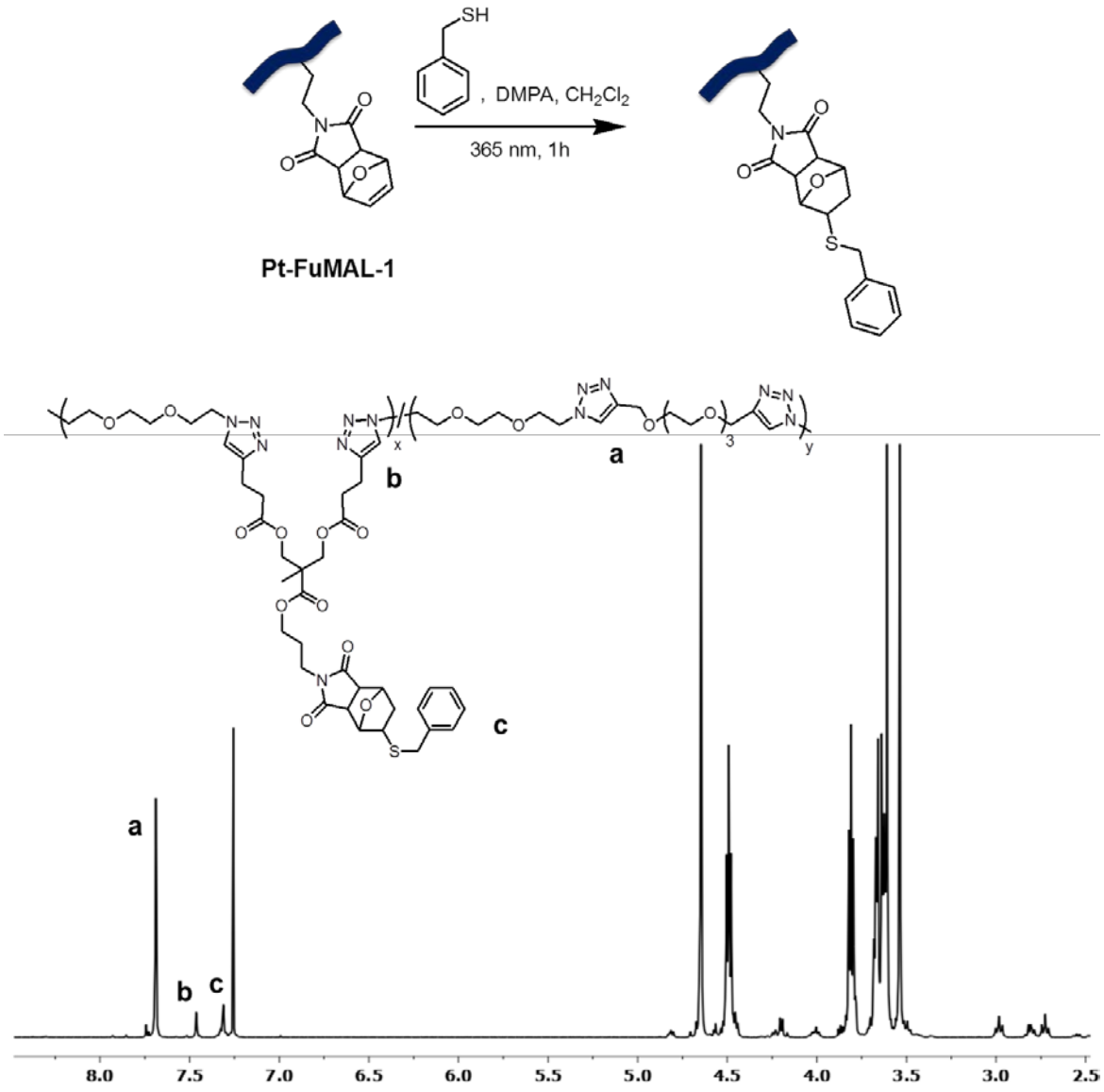


Figure 6.13. Synthetic transformation and ^{1}H NMR spectrum of radical thiol-ene functionalized copolymer Pt-FuMAL-1.

6.3.3. Synthesis of Thiol-reactive Hydrogels via Michael-type Thiol-ene Reaction

Covalent crosslinking of copolymer containing maleimide side groups was investigated using a bis-thiol bearing crosslinker. A determined amount of the copolymer Pt-MAL-1 and slightly lower amount of bis-thiol compound 2,2′-(ethylenedioxy) diethanethiol than the calculated maleimide ratio were dissolved in dichloromethane in separate vials. After mixing two solutions, the hydrophilic backbone of polytriazole yielded hydrogel structure via tandem crosslinking based on Michael addition of thiols to maleimide groups (Figure 6.14). A catalytic amount of triethylamine was used to accelerate the reaction. The gel formation was rapid and after about one minute there was no flow of sample. After hydrogel formation, unreacted species were removed by washing the gel with THF followed by distilled water several times. The swollen hydrogel sample was frozen and freeze-dried in vacuo to yield dried hydrogel.

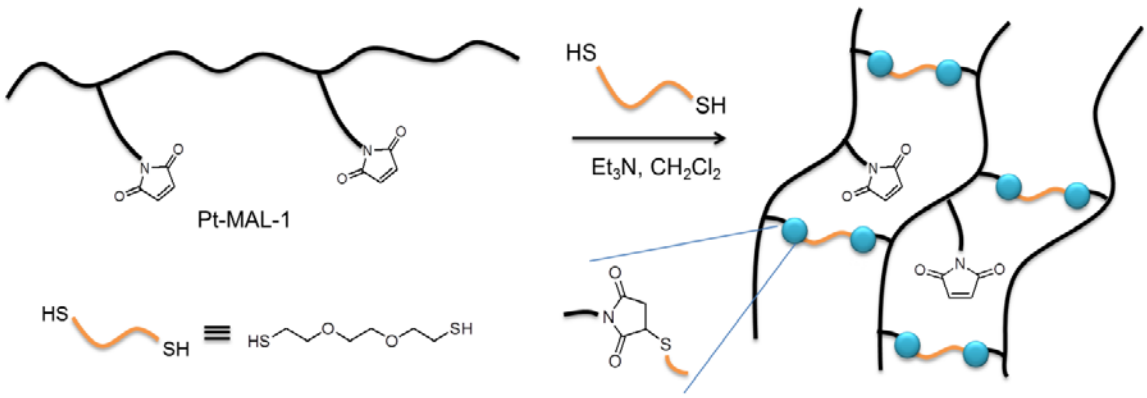


Figure 6.14. General reaction scheme of hydrogel formation.

The obtained hydrogel was characterized with scanning electron microscopy (SEM) to reveal a microstructure characteristic of a rubber-like randomly cross-linked polymeric material (Figure 6.15). As expected, the PEG-based hydrogel exhibits excellent water uptake (1200 % by weight) and reaches swelling equilibrium within 30 minutes (Figure 6.16).

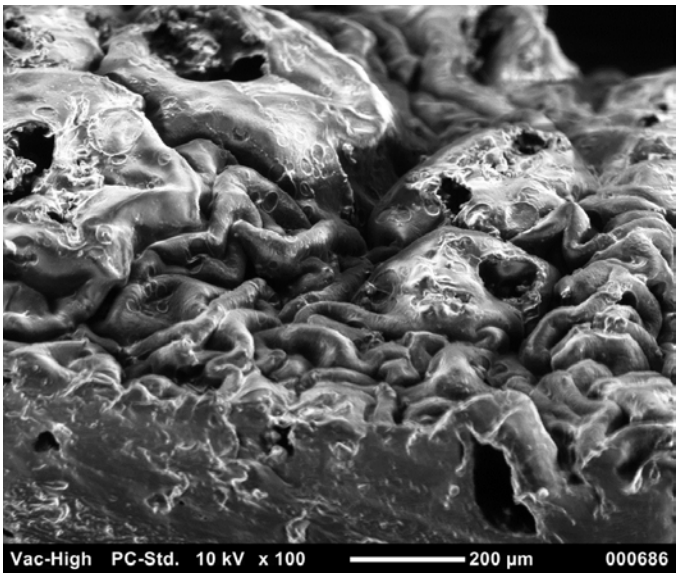


Figure 6.15. SEM image of hydrogel synthesized from copolymer Pt-MAL-1.

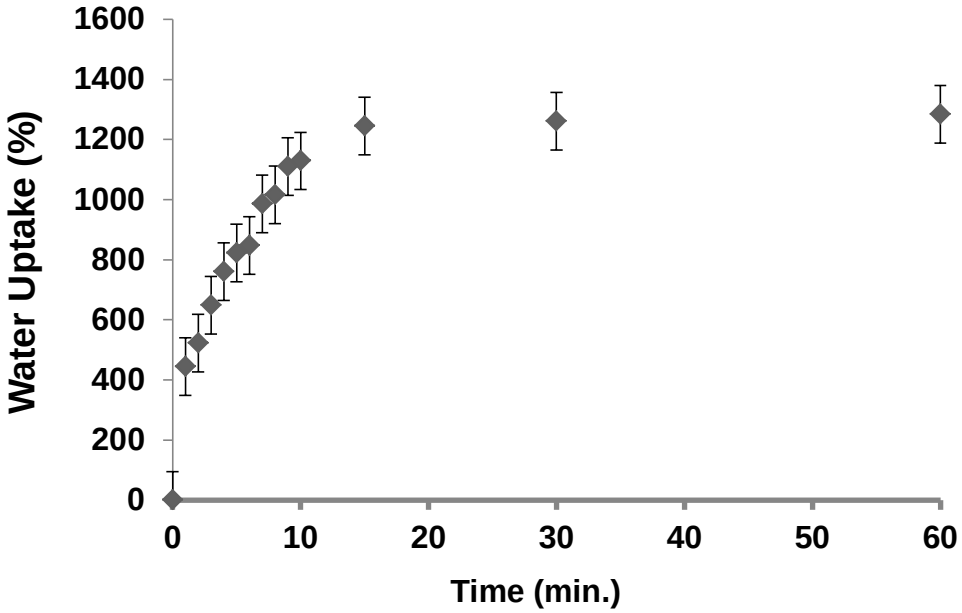


Figure 6.16. Swelling profile of hydrogel.

Functionalization of the residual free maleimide groups in the hydrogel was investigated by conjugation of a thiol-containing fluorescent dye, namely, SAMSA fluorescein (Figure 6.17a). Hydrogel was incubated with excess of dye in water for 3 h. After purification, the extent of conjugation was studied by fluorescence microscopy. As shown in fluorescence microscopy image in Figure 6.17b, thiol containing dye was efficiently attached to hydrogel. As a control experiment, the maleimide containing

hydrogels were treated with acetyl-protected sulfhydryl-bearing fluorescein dye; and as expected, no fluorescence was observed due to the lack of any covalent conjugation of the dye to the hydrogel. Thus, the pendant maleimide groups in these PEG-based copolymers could serve the dual role of enabling fabrication of hydrogels under mild conditions as well as their facile functionalization with other thiol-containing molecules of interest.

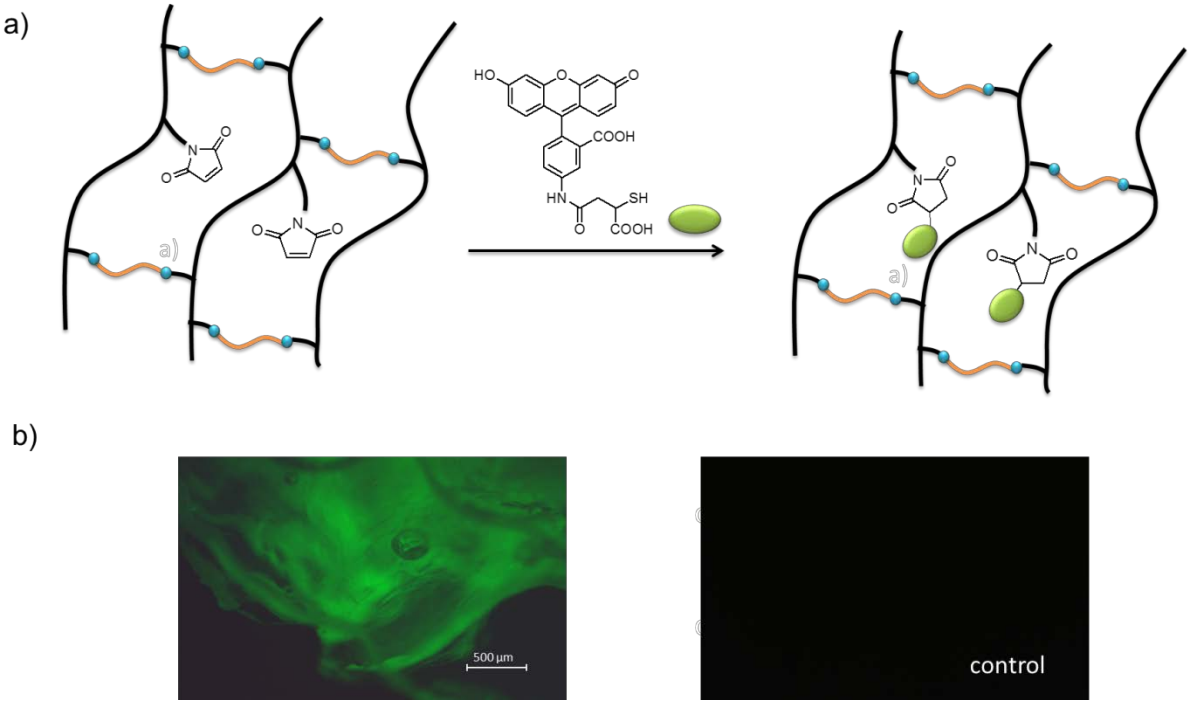


Figure 6.17. a) General representation of hydrogel functionalization b) Fluorescence microscope image of dye-functionalized hydrogel.

6.4. Conclusion

Thiol-reactive copolymers based on linear polytriazoles with oligo(ethylene glycol) backbone appended with maleimide functional groups as side chains were prepared using step-growth polymerization. The copper(I)-catalyzed Huisgen-type 1,3-dipolar azide-alkyne cycloaddition reaction was utilized for the copolymerization of diazide and dialkyne functionalized monomers. Copolymerization of triethylene glycol derivatives were first investigated using different copper(I) catalysts to determine optimum reaction conditions and catalyst system. Thereafter, a diyne functionalized furan-protected maleimide containing monomer was incorporated into copolymers with varying feed ratios. The maleimide groups along the side chain of the copolymers were unmasked via

the retro-Diels-Alder reaction. Obtained copolymers were functionalized via nucleophilic thiol-ene and Diels-Alder reactions. Additionally the furan-protected copolymer could be functionalized using photo-initiated radical thiol-ene reaction. These copolymers could be covalently cross-linked using bis-thiol based crosslinkers under mild conditions to yield hydrogels. By adjusting the relative stoichiometry of the thiol and maleimide functional groups during the crosslinking, hydrogels containing residual maleimide groups can be obtained. These maleimide containing hydrogels undergo facile functionalization using thiol-containing small molecules such as fluorescent dyes. The approach outlined in this work can be extended to obtain various reactive PEG-based polytriazole-containing copolymers for appropriate post-polymerization functionalization through judicious choice of monomer building blocks.

7. BIO-INSPIRED ANCHORABLE THIOL-REACTIVE POLYMERS: SYNTHESIS AND APPLICATIONS TOWARDS SURFACE FUNCTIONALIZATION OF MAGNETIC NANOPARTICLES

The materials in this chapter have been adapted with modifications from the following article: Arslan M., T.N. Gevrek, J. Lyskawa, S. Szunerits, R. Boukherroub, R. Sanyal, P. Woisel, A. Sanyal, *Macromolecules*, Vol. 47, pp. 5124-5134, 2014.

7.1. Introduction

Polymeric coatings are mixtures of film-forming materials derived from various polymers to yield thin films on solid substrates. These materials are usually applied on surfaces to provide adherence and protection as well as functional and decorative purposes [170]. A wide range of materials that polymer-based coatings could be applied include metal surfaces, ceramics and synthetic materials. Polymeric coatings are needed to finely adhere to the applied substrate and are required to not chip easily. They are also required to show stability against heat, moisture and chemicals.

By the advancements in new fabrication and characterization techniques, preparation and applications of nanoscaled materials has gained increased interest in a few decades [171]. Compared to the bulk counterparts, nano-sized materials exhibit unique optical, magnetic and electrical properties. Due to these new features, nanoparticles find extensive applications in technological, environmental, biomedical requests as well as energy applications (Figure 7.1).

Figure 7.1. Application areas of nanoparticles (From [172]).

In biomedical applications, commonly utilized nanoparticle forms include inorganic, metallic, magnetic, polymeric nanoparticles, protein-based nanoparticles and quantum dots [173]. In magnetic nanoparticles, iron oxide-based nanoparticles (IONPs) are beyond the most widely investigated nanoparticles that find wide range of applications in biomedical fields (Figure 7.2). These applications include magnetic resonance imaging (MRI) [174], drug delivery [175], biomolecular separation (separation proteins, enzymes and DNA) [176], and stem cell tracking [177].

For most of the practical applications of nanoparticles, surface coating or chemical modification with various substrates is usually accounted. The purpose of surface modification may include nanoparticle stabilization, functionalizing the nanoparticles with related ligands or promoting the nanoparticle assembly [178].

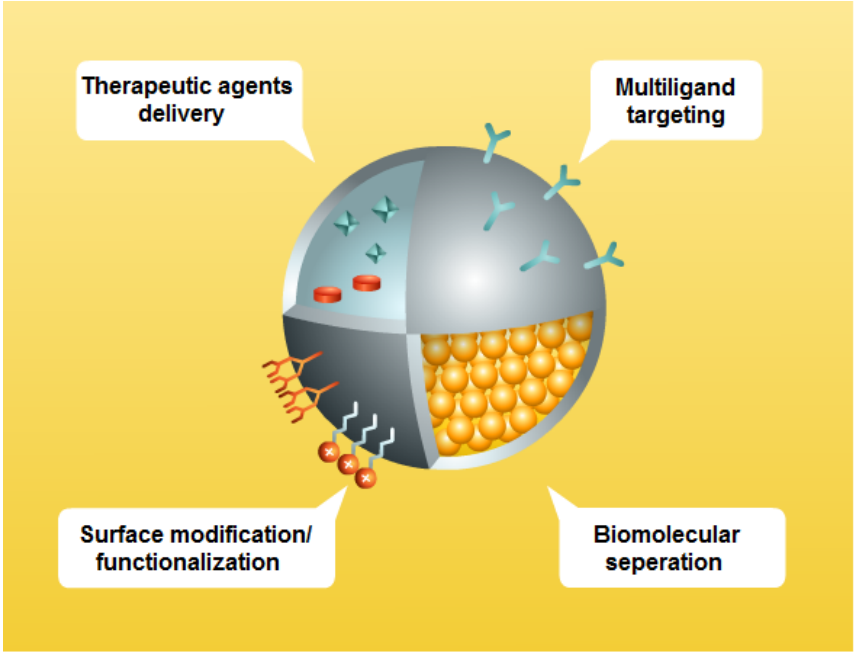


Figure 7.2. Application areas of iron oxide nanoparticles in biomedical fields (Adapted from [172]).

Polymeric coatings with functionalities on solid surfaces are widely utilized to fabricate interfaces with properties and characteristics that enable them to interact with their environment in a desired manner. For instance, the coating can be designed to diminish undesirable interactions, or augment desired interactions, and oftentimes do both of these simultaneously in a selective fashion. This balancing act is necessary for many practical applications such as biomolecular sensing and immobilization. For example, the receptor sites on a sensor surface should have specificity for a particular biomolecule and simultaneously present an antifouling surface to other biomolecules to minimize non-specific absorption and thus increase device sensitivity. Polymeric coatings are ideal candidates for such applications since polymers can be readily synthesized to be multifunctional by incorporation of several monomers with different properties and functions.

Polymeric coatings containing reactive functional groups that can be modified under mild metal-free reaction conditions are attractive for various applications that involve immobilization of ligands, peptides and biomolecules. It is important that the reactive polymers can be anchored onto surfaces to provide a stable coating. A variety of anchoring groups have been investigated for a wide variety of surfaces.

Recently, much attention has been focused upon the catechol functional group as it is found to be a component of glues utilized by nature. Catechol functional group containing dopamine residues were found in the protein components of the byssus threads that mussels utilize for anchoring onto a diverse array of surfaces and was identified to be responsible for their strong adhesive properties [176, 177].

Since then, catechol functional group has been widely used in as a building block in the fabrication of various polymeric materials [178–180]. Polymeric materials containing catechol functional groups have been utilized in surface modifications of nanoparticles and planar surfaces [184], fabrication of adhesive fibers and wound dressings (Figure 7.3). Recent reports show successful implementation of catechol containing copolymers for effective modification of nanoparticles and surfaces to impart them with anti-biofouling and antibacterial properties. In particular, catechol grafted poly(ethylene glycol) and phosphorylcholine based hydrophilic polymers have been used for surface modifications in a materials-independent manner to impart anti-biofouling characteristics [182, 183].

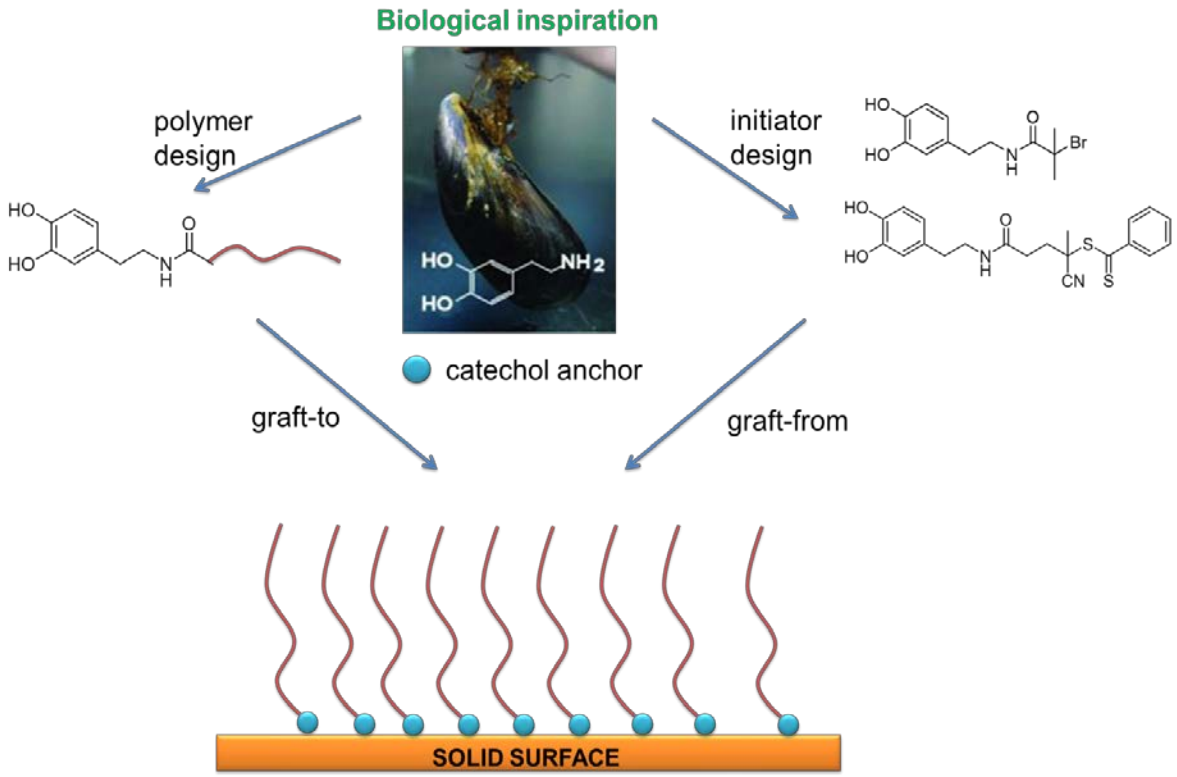


Figure 7.3. Biologically inspired polymer grafting based on catechol anchor groups.

Incorporation of reactive functional groups along with the catechol based surface adhesive groups into the same polymeric chain yields copolymers that can be used towards modification of surfaces with reactive coatings. Towards this end, various copolymers containing both catechol group and functionalizable groups were synthesized and utilized as reactive coatings [147]. We hypostatize that polymers carrying adhesive catechol groups along with thiol-reactive side chains that can undergo metal-free conjugation reactions can be considered as attractive platforms as functionalizable surface coatings. Such polymers will be amenable for surface modifications of iron oxide and titanium nanoparticles and planar surfaces and allow subsequent functionalization of these surface tethered polymers with thiol-containing molecules for desired applications.

In this study, we report the synthesis of novel thiol-reactive polymers containing a catechol moiety and demonstrate their application towards surface modification of magnetic nanoparticles [187]. Poly(oligo(ethylene glycol) acrylate) (PEGMEA) based hydrophilic polymers incorporating maleimide group containing side-chains were synthesized using reversible addition fragmentation chain transfer (RAFT) polymerization. A furan-protected maleimide-containing acrylate monomer was synthesized and copolymerized with hydrophilic monomers in the presence of a catechol moiety containing chain transfer unit. Removal of the furan protecting group after polymerization yields polymers that are thiol-reactive and possess an intact catechol unit at chain-end for anchoring onto various surfaces. Oleic-acid coated organo-dispersible magnetic nanoparticles were modified with the hydrophilic catechol terminated polymers to obtain water dispersible polymer-coated magnetic nanoparticles (Figure 7.4). The reactive maleimide units on the polymer-coated nanoparticles were utilized to conjugate thiol-containing hydrophobic dye using the nucleophilic Michael addition reaction to obtain water dispersible magnetic and fluorescent nanoparticles. Alternatively, it was demonstrated that radical thiol-ene reaction can also be utilized to functionalize nanoparticles coated with polymers where the maleimide group is in masked as a furan-cycloadduct. Efficient anchoring of the polymer onto nanoparticle surfaces and their subsequent functionalization was established using various techniques such as infrared spectroscopy (IR), thermogravimetric analysis (TGA) and phase transfer studies, whereas the subsequent functionalization was evident from the organic to aqueous phase transfer of the hydrophobic fluorescent dye.

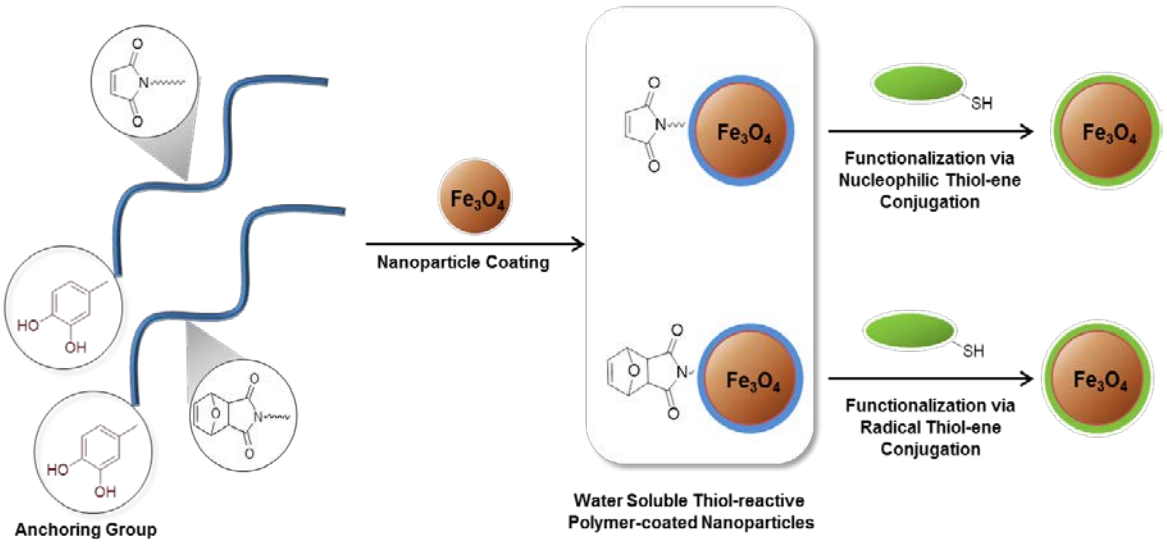


Figure 7.4. Schematic illustration of fabrication and functionalization of thiol-reactive magnetic nanoparticles derived from a hydrophilic anchorable and reactive copolymer.

7.2. Experimental

7.2.1. Materials and Characterization

Poly(ethylene glycol) methyl ether acrylate (PEGMEA, M_n = 454), was purchased from Sigma Aldrich and purified by passing through the activated aluminum oxide column prior to use. Acryloyl chloride, triethylamine and 2,2-dimethoxy-2-phenylacetophenone (DMPA) were purchased from Sigma Aldrich and used as received. 2,2′-Azobis(2-methylpropionitrile) (AIBN) was purchased from Sigma Aldrich and recrystallized from methanol before use. Dichloromethane and methanol were purchased from Merck. Anhydrous toluene was obtained from SciMatCo purification system. Column chromatography was performed using silica gel 60 (43-60 nm, Merck). Thin layer chromatography was performed using silica gel plates (kieselgel 60 F254, 0.2 mm, Merck). The plates were viewed under 254 nm UV lamp. 4,4-Difluoro-1,3,5,7-tetramethyl-8-[(10-mercapto)]-4-bora-3a,4a-diaza-s-indacene (BODIPY-SH) was synthesized according to literature procedure [188]. Furan-protected maleimide containing alcohol (1) [108], and catechol-containing CTA (Dopa-CTA) [189] were prepared according to previously reported literature protocols. Elemental analysis data on monomer was obtained using a Thermo Electron S.p.A Flash EA® 1112 elemental analyzer. The molecular weights were determined by gel permeation chromatography analysis using a Viscotek GPCmax VE-

2001 analysis system. PLgel (length/ID 300 mm x 7.5 mm, 5 µm particle size) Mixed-C column was calibrated with polystyrene standards (1–150K) using refractive index detector. THF was used as the eluent at a flow rate of 1 mL/min at 30 ^{o}C. Characterization of monomer and copolymers were performed using ^{1}H NMR, ^{13}C NMR spectroscopy (Varian 400 MHz) and attenuated total reflectance-Fourier transform infrared spectroscopy (Thermo Fisher Scientific Inc.; Nicolet 380). Thermogravimetric analysis (TGA) was carried out on a TA Instruments at a heating rate of 10 ^{o}C/min under nitrogen atmosphere. UV studies were performed with a Varian Cary 50 Scan UV/vis spectrophotometer. All electrochemical experiments (cyclic voltammetry) were performed using an Autolab PGSTAT 30 workstation. The electrolyte solution (0.05 M) was prepared from recrystallized tetrabutylammonium hexafluorophosphate (Bu_4NPF_6) and dry acetonitrile or dry dichloromethane. A three-electrode configuration was used with a platinum disk (2 mm diameter) as working electrode, with an Ag/AgCl reference electrode and a platinum wire as the counter electrode. All measurements were recorded at 20 ^{o}C under a nitrogen atmosphere. The solution was purged with nitrogen prior to electrochemical analyses. Oleic acid coated magnetic nanoparticles were synthesized according to previously reported literature using the coprecipitation method [190]. Particle sizes of synthesized and modified magnetic nanoparticles were determined using a Delong LVEM5 low voltage electron microscope in the transmission mode. Samples were dropped on grid from a THF suspension. The average diameter and polydispersity index of the magnetic nanoparticles before and after coating with polymers were determined by dynamic light scattering using a Zetasizer@Nano ZS (Malvern Instruments S. A., Worcestershire, U.K.). All nanoparticle suspensions were diluted at 1/100 (v/v) in distilled water (filtered over 0.22 mm) prior to analysis and analyzed in triplicate.

7.2.2. Synthesis of Furan-protected Maleimide-containing Acrylate Monomer (FuMA)

Under nitrogen, acryloyl chloride (0.38 mL, 4.7 mmol) was added dropwise to a mixture of alcohol 1 (1.00 g, 4.43 mmol) and triethylamine (0.52 mL, 5.31 mmol) in dichloromethane (100 mL). The resulting solution was stirred 3 h at 0 ^{o}C and subsequently at ambient temperature overnight. After completion of the reaction as indicated by TLC, the reaction mixture was washed with saturated $NaHCO_3$ (2 x 40 mL) and H_2O (2 x 40 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo.

Obtained yellow residue was purified by column chromatography (EtOAc:Hexane 1:1) to yield 1.21 g (92 % yield) of pure crystalline product. ^{1}H NMR ($CDCl_3$, δ, ppm) 6.48 (s, 2H, CH=CH), 6.39 (dd, J_1 = 17.4 Hz, J_2 = 1.4 Hz, 1 H), 6.14-6.07 (m, 1H), 5.81 (dd, J_1 = 10.6 Hz, J_2 = 1.4 Hz, 1 H), 5.24 (s, 2H, CH bridgehead protons), 4.11 (t, 2H, J = 6.0 Hz, OCH_2), 3.58 (t, 2H, J = 6.8 Hz, NCH_2), 2.81 (s, 2H, CH-CH bridgehead protons), 1.98-1.91 (m, 2H, $NCH_2CH_2CH_2O$). ^{13}C NMR ($CDCl_3$, δ, ppm) = 176.23, 166.12, 136.62, 131.02, 128.37, 81.05, 61.63, 47.51, 35.95, 26.79. Anal. Calcd. [$C_{14}H_{15}NO_5$]: C, 60.64; H, 5.45; N, 5.05. Found: C, 60.23; H, 5.72; N, 4.89.

7.2.3. Typical Procedure for Copolymerization of Monomer FuMA with PEGMEA using RAFT Polymerization

An example RAFT polymerization procedure of FuMAP2 was as follows. PEGMEA (210.0 mg, 0.45 mmol), FuMA monomer (14.4 mg, 0.05 mmol), Dopa-CTA (6.0 mg, 0.015 mmol) and AIBN (0.5 mg, 0.003 mmol) were placed in a sealed tube equipped with a magnetic stir bar. The mixture was purged with nitrogen for 15 min before nitrogen-purged toluene (1 mL) was added. The reaction mixture was stirred at 70 °C for 16 h. After polymerization, the solvent was removed under reduced pressure and remaining crude polymer was re-dissolved in minimum amount of dichloromethane before precipitating into the cold ether (repeated twice). Obtained polymer was dried under vacuum.

7.2.4. Typical Procedure for Post-polymerization Activation of Maleimide Functional Groups

Polymer FuMAP2 (100 mg) was dissolved in anhydrous toluene (150 mL) and refluxed under a nitrogen atmosphere. After 8 h, the solution was cooled to room temperature and solvent was evaporated in vacuo to yield polymer MAP2 in quantitative yield. Thus obtained polymer was characterized using SEC and ^{1}H NMR.

7.2.5. Modification of Iron-oxide Nanoparticles using Polymer MAP2

Oleic-acid coated magnetic nanoparticles (20 mg) and polymer MAP2 (100 mg) were dispersed into chloroform (2 mL) and heated to 40 ^{o}C under nitrogen atmosphere for 16 h. Thereafter solvent was evaporated and particles were dispersed in THF. Particles were isolated by centrifugation at 5000 rpm for 15 minutes. Isolation using magnet was

carried out. Polymer coated nanoparticles were dispersible in water and stable for several weeks. Samples for analysis using FT-IR and TGA were dried under vacuum before measurements.

7.2.6. Conjugation of BODIPY-SH onto Polymer coated Magnetic Nanoparticles via Radical-Thiol-Ene Reaction

Polymer FuMAP2 coated magnetic nanoparticles (10 mg) were dispersed in THF (0.2 mL) and BODIPY-SH (0.25 mg) and DMPA (0.03 mg) was added to this solution. The solution was incubated under UV light (λ_{ex} = 365 nm) for 30 minutes. Dye-conjugated particles were isolated and purified from excess dye by centrifugation in THF several times.

7.2.7. Conjugation of BODIPY-SH onto Polymer coated Magnetic Nanoparticles via Nucleophilic Thiol-Ene Reaction

Polymer MAP2 coated magnetic nanoparticles (10 mg) were dispersed in THF (2 mL) and BODIPY-SH (0.25 mg) was added to this solution. The solution was incubated at 40 ^{o}C under nitrogen atmosphere for 16 h. After conjugation, particles were isolated and purified from excess dye by centrifugation in THF several times.

7.3. Results and Discussion

7.3.1. Synthesis of Catechol Chain-end Functionalized Maleimide-containing Copolymers

A novel furan-protected maleimide functional group containing monomer (henceforth referred to as FuMA-monomer) was synthesized and copolymerized using RAFT polymerization in the presence of a catechol containing CTA (Figure 7.5). The FuMA-monomer was synthesized by treatment of furan-protected maleimide containing alcohol with acryloyl chloride in the presence of triethylamine at room temperature in 92 % yield.

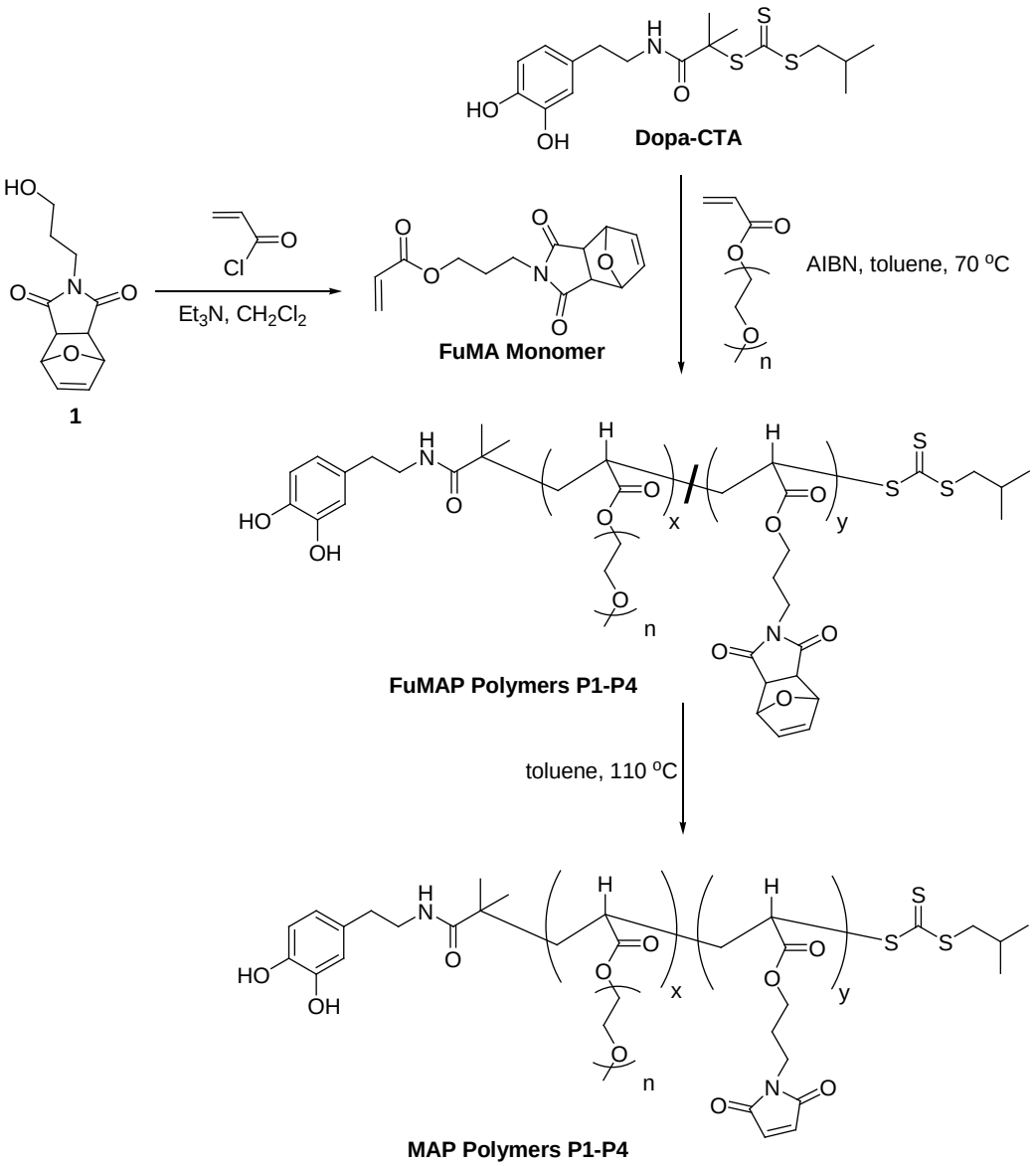


Figure 7.5. Synthesis of catechol chain-end functionalized thiol-reactive maleimide side-chain containing copolymers P1-P4.

Pure FuMA-monomer could be readily obtained in multi gram quantities after column chromatography using SiO_2 as stationary phase. The ^{1}H NMR spectrum of the monomer shows the presence of the bicyclic structure from proton resonances at 6.48, 5.24 and 2.81 ppm (Figure 7.6). The presence of the polymerizable acrylate unit is evident from resonances belonging to the vinylic hydrogen atoms at 6.39, 6.14-6.07 and 5.81 ppm. Analysis of monomer FuMA using ^{13}C NMR (Figure 7.7) and elemental analysis further established its identity and purity.

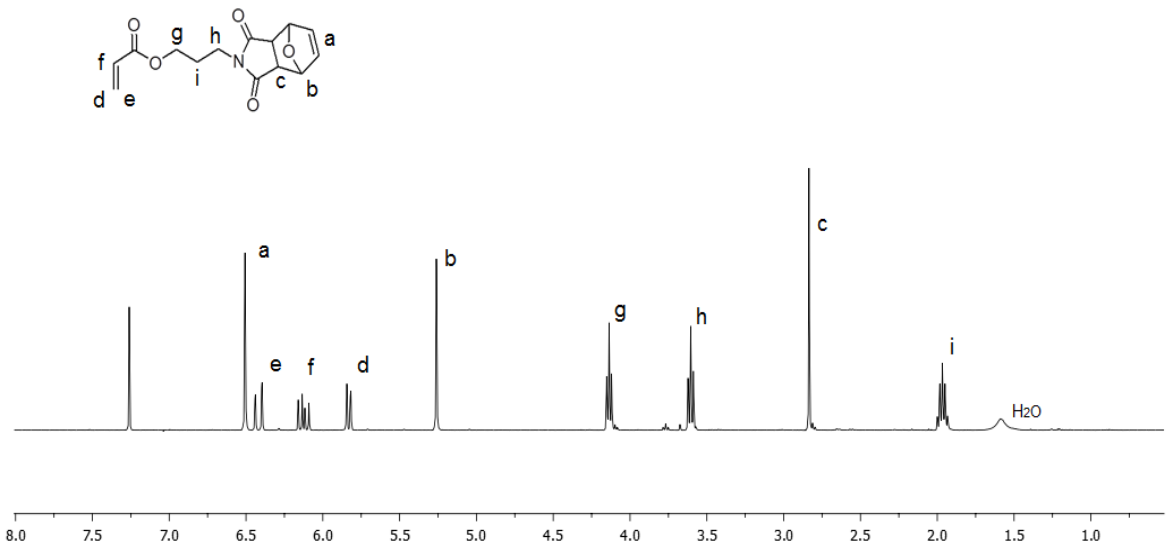


Figure 7.6. ^{1}H NMR spectrum of the acrylate-based FuMA monomer containing the furan-protected maleimide functional group.

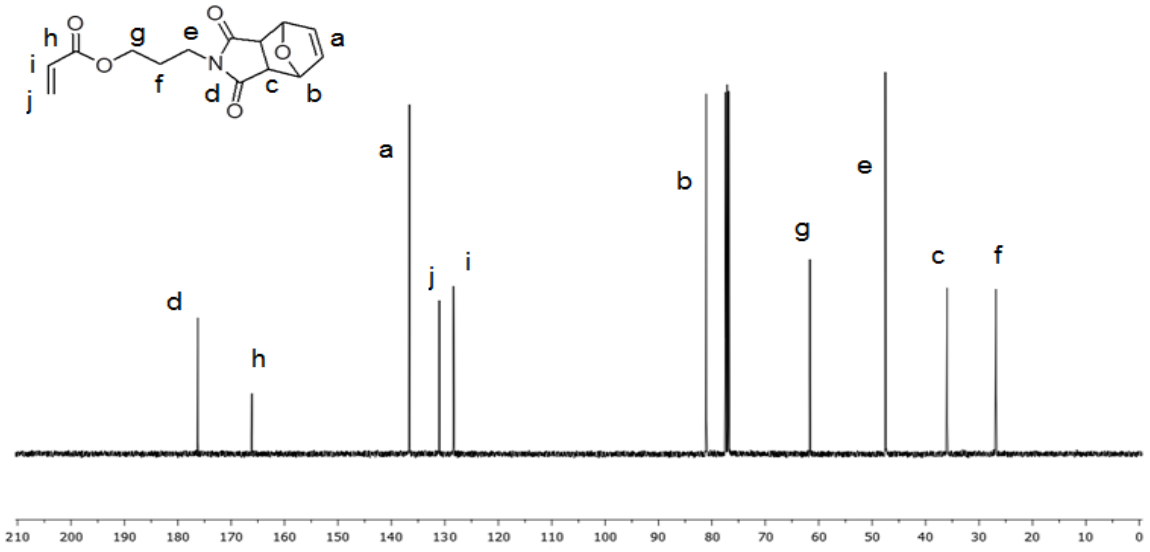


Figure 7.7. ^{13}C NMR spectrum of the acrylate-based FuMA-monomer containing the furan-protected maleimide functional group.

Polymers FuMA(P1-P4) with varying amounts of FuMA-monomer were synthesized by copolymerization with a hydrophilic monomer PEGMEA using RAFT polymerization in the presence of catechol-containing Dopa-CTA in toluene at 70 ^{o}C (Table 7.1, Items 1-4). The relatively low ratio between [CTA]/[AIBN] (5/1) was considered to be moderate to reduce the amount of polymer derived from the initiator still allowing the control on polymerization. During the polymerization, the temperature was kept low enough to avoid any retro Diels-Alder reaction mediated deprotection of the masked monomer yet high

enough to obtain polymers with intended molecular weights within reasonable reaction times. Polymers were obtained in pure form after removal of unreacted monomers upon their precipitation with diethyl ether.

Table 7.1. Polymerization Conditions and Copolymers Characterizations Before and After Unmasking the Maleimide Functional Group.

Item	Polymer[a]	$F_{theo.}$[b] (%)	$F_{calc.}$[c] (%)	Conv. (%)	$M_{n,theo.}$ (g/mol)	$M_{n,NMR}$[c] (g/mol)	$M_{n,SEC}$[d] (g/mol)	M_w/M_n[d] (g/mol)
1	FuMAP1	5 : 95	2.7 : 97.3	73	10130	11900	7780	1.20
2	FuMAP2	10 : 90	6.7 : 93.3	67	9160	7470	7670	1.17
3	FuMAP3	20 : 80	13.6 : 86.4	65	8560	9850	6450	1.21
4[e]	FuMAP4	30 : 70	18.4 : 81.6	42	5450	5480	6680	1.26
5	CP1	0 : 100	-	77	11530	12460	8360	1.23
6	CP2	0 : 100	-	69	9350	7890	6870	1.14

[a] $[M]_o$:$[I]_o$:150; $[I]_o/[CTA]_o$: 1/5; $[M]_o$: 0.5 M; CTA: Dopa-CTA for FuMA(P1-P4) and CP2, 2-cyano-2-propyl dodecyl trithiocarbonate for CP1; Reaction time 16 h, 70 °C, solvent toluene. [b] $F_{theo.}$: [FuMA]:[PEGMEA]. [c] Determined by ^{1}H NMR. F_{calc}: [FuMA]:[PEGMEA]. [d] Estimated by SEC eluted with THF, using polystyrene standards. [e] $[M]_o$: 0.1 M.

Analysis of these copolymers using ^{1}H NMR revealed successful incorporation of the FuMA-monomer into the copolymers, as was evident from the proton resonances around 6.51, 5.23 and 2.86 ppm due to the bicyclic moiety of the monomer. The presence of the catechol group at the chain end was established by proton resonances belonging to the phenyl ring in the ^{1}H NMR of the copolymers (Figure 7.8). The presence of the trithiocarbonyl unit from the chain transfer agent was evident from the presence of characteristic absorbance at λ= 308 nm in the UV-vis spectrum of the copolymer (Figure 7.9). The extent of incorporation of the monomers into the final copolymers for particular feed ratios were obtained by comparison of integration of the proton resonance at 3.37 ppm due to the terminal methyl ether group of the PEGMEA monomer with the vinylic protons from the bicyclic structure of the FuMA-monomer at 6.51 ppm. The experimentally obtained monomer compositions of the copolymers show that the incorporation of the masked monomer is lower than aimed in the feed (Table 7.1, Items 1-4). This discrepancy can be attributed to the steric hindrance of the pendant bicyclic group in the monomer. Analysis of these copolymers using size exclusion chromatography established that

copolymers with good control over molecular weight and narrow polydispersities were obtained (Table 7.1 and Figure 7.10).

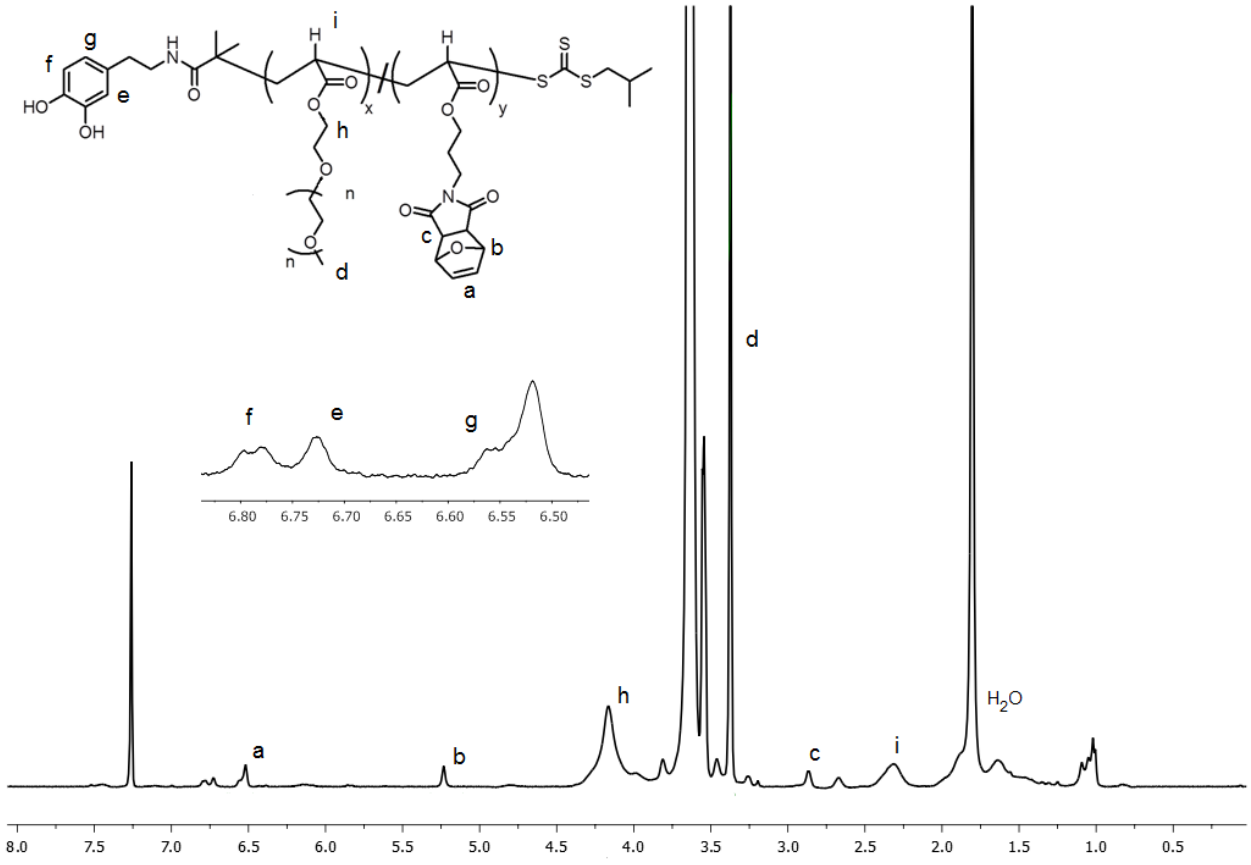


Figure 7.8. Representative ^{1}H NMR spectrum of catechol end-functionalized furan protected maleimide group containing copolymer FuMAP2.

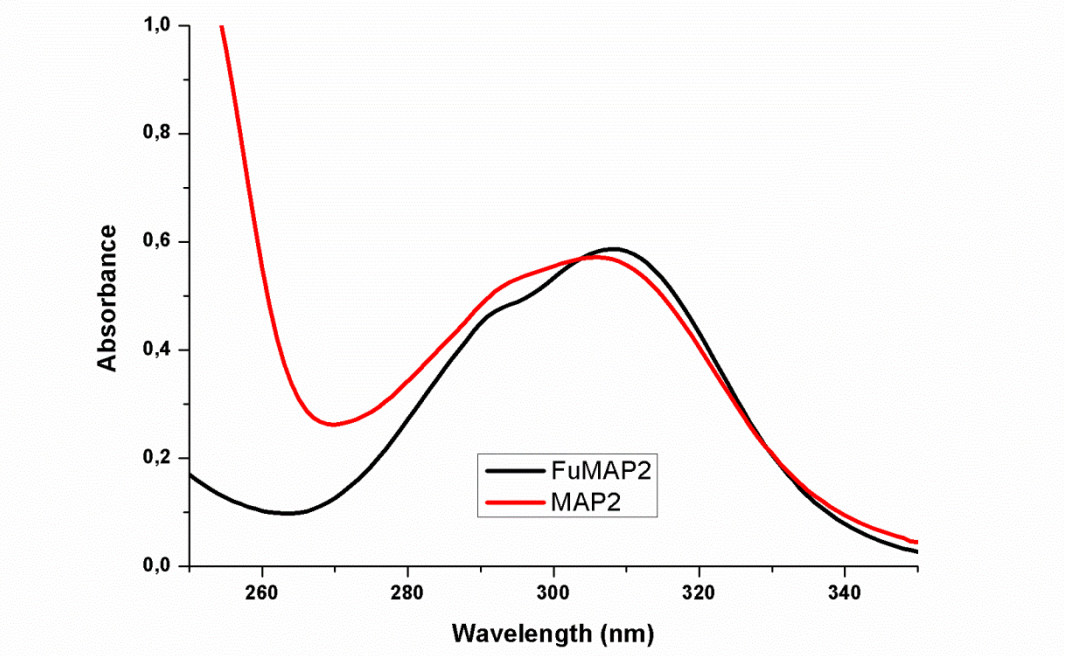


Figure 7.9. UV-vis spectra of catechol end-functionalized maleimide group containing copolymers FuMAP2 and MAP2.

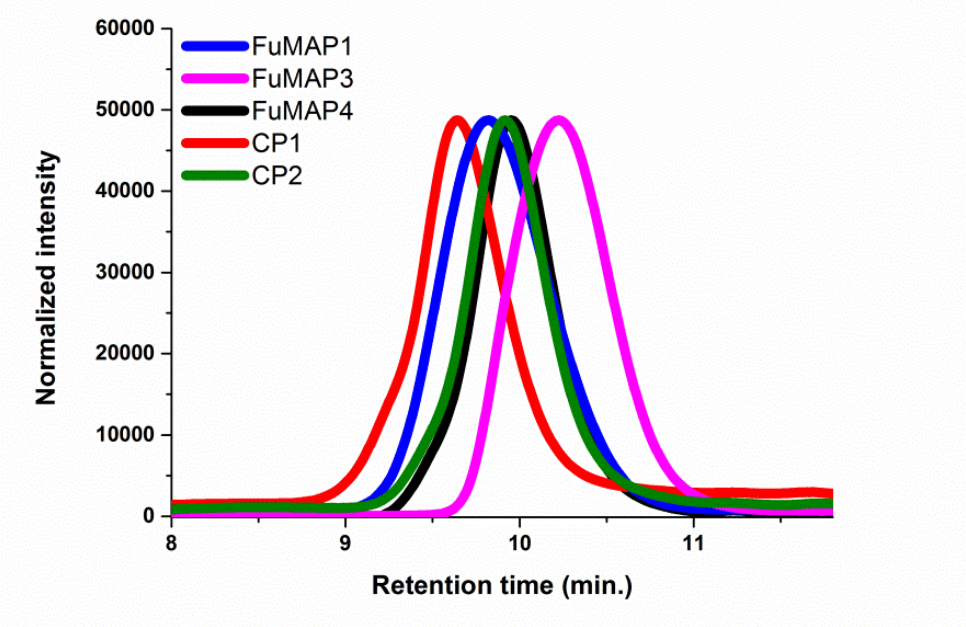


Figure 7.10. SEC traces of copolymers.

The polymers were subjected to retro Diels-Alder reaction to obtain the maleimide units in their thiol-reactive form by removal of the furan protecting group. Polymers were dissolved in anhydrous toluene and refluxed at 110 ^{o}C to unmask the maleimide groups. Analysis of the obtained polymers using ^{1}H NMR spectroscopy revealed that the removal

of the furan protecting groups takes place in a quantitative fashion (Figure 7.11). Complete disappearance of the proton resonances of the bridgehead bicyclic structure and appearance of a new peak at 6.75 ppm due to the maleimide group was observed. There is a good agreement between the integration values of catechol protons and pendant PEGMEA monomer methoxy protons before and after the retro Diels-Alder reaction confirming the conservation of Dopa groups at side chains.

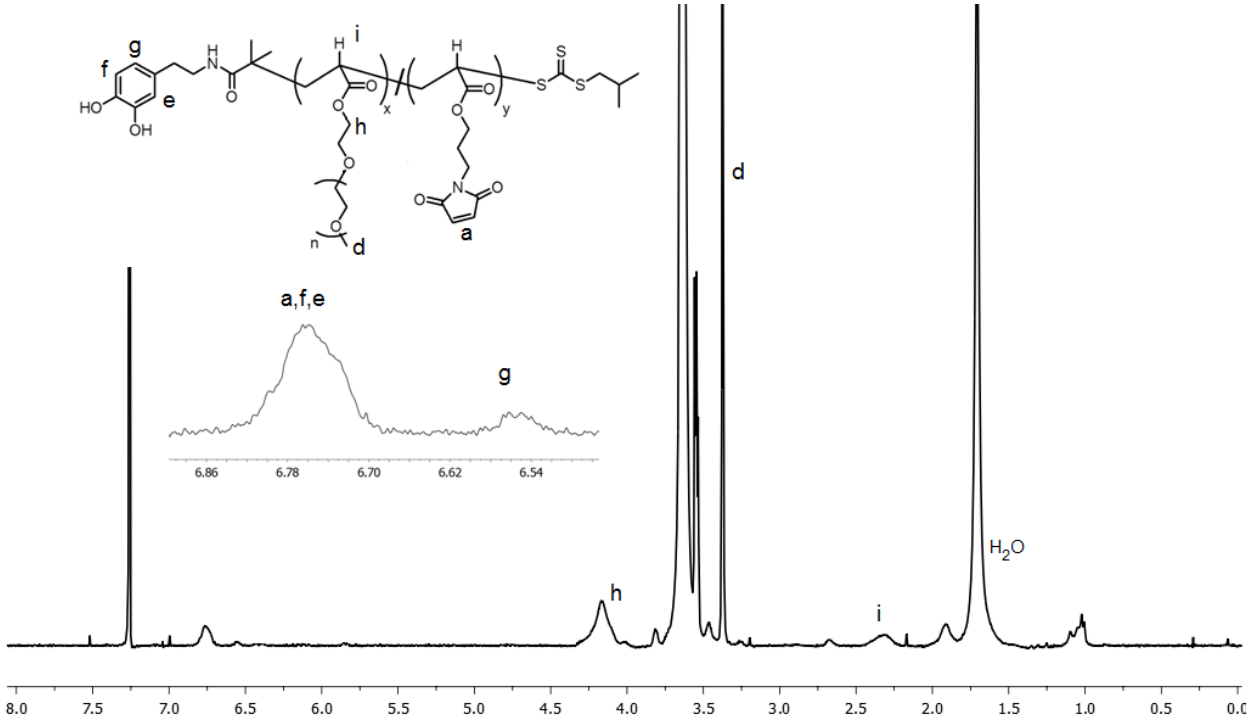


Figure 7.11. ^{1}H NMR spectrum of catechol terminated unmasked maleimide containing copolymer MAP2.

Analysis using size-exclusion chromatography revealed that no significant change in the molecular weight and its distribution occurs for the polymers after the retro Diels-Alder step, suggesting that the thermal post-polymerization modification does not have any deleterious effect on the polymers structure (Figure 7.12). Furthermore, cyclic voltammetry experiments were carried out in acetonitrile to unequivocally establish the presence of the electroactive catechol group at the polymer chain-end (Figure 7.13). CV of end-decorated catechol copolymer MAP2 gave rise to the irreversible two-electron oxidation wave around 1.1 V vs Ag/AgCl, originating from the oxidation of the catechol unit into its corresponding quinone [189]. Furthermore, no significant shift in the position of the oxidation wave of MAP2 compared to Dopa-CTA was observed suggesting that the

polymer chain does not affect the redox properties of the catechol unit. No significant change in absorption due to the trithiocarbonyl moiety in the UV-Vis spectrum of the copolymer MAP2 indicated that the trithiocarbonyl group at the chain end remained preserved during the thermal activation of the maleimide group (Figure 7.13).

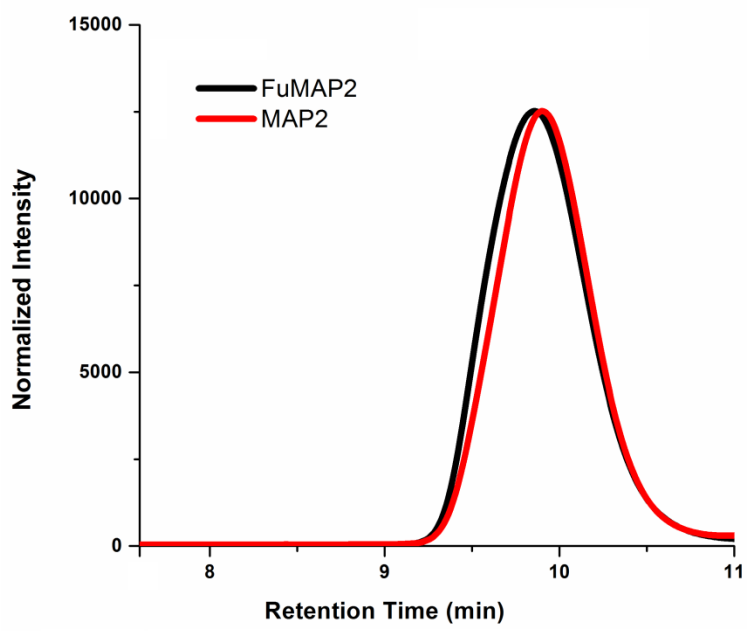


Figure 7.12. Size-exclusion chromatography (SEC) traces of copolymer FuMAP2, before and after retro Diels Alder reaction.

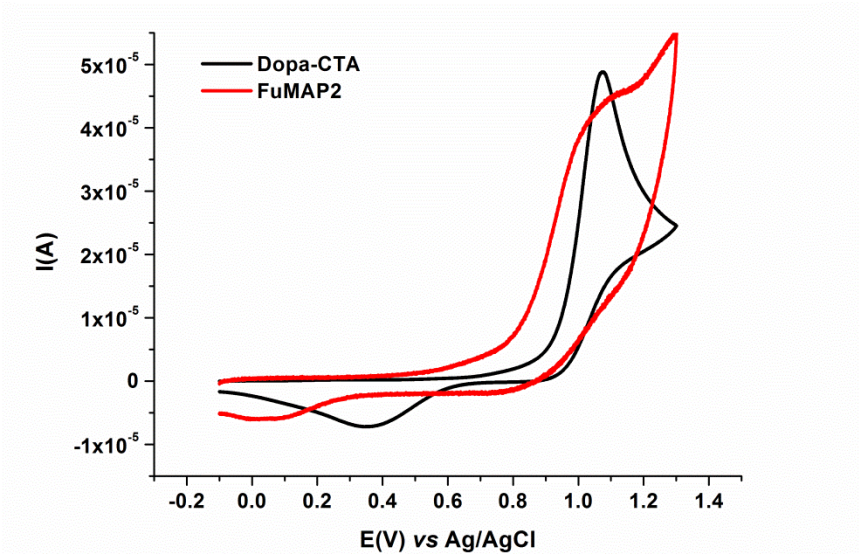


Figure 7.13. Cyclic voltammetry plots of dopa-CTA and catechol end-functional copolymer FuMAP2.

The removal of the furan protecting group via the cycloreversion step was also probed using thermogravimetric analysis (TGA). The weight loss observed for the copolymers between temperatures of 90 and 150 ^{o}C is due to release of furan correlated well with the expected values. As expected, an increase in the value of weight loss was observed for copolymers with higher content of the furan protected monomer (Figure 7.14).

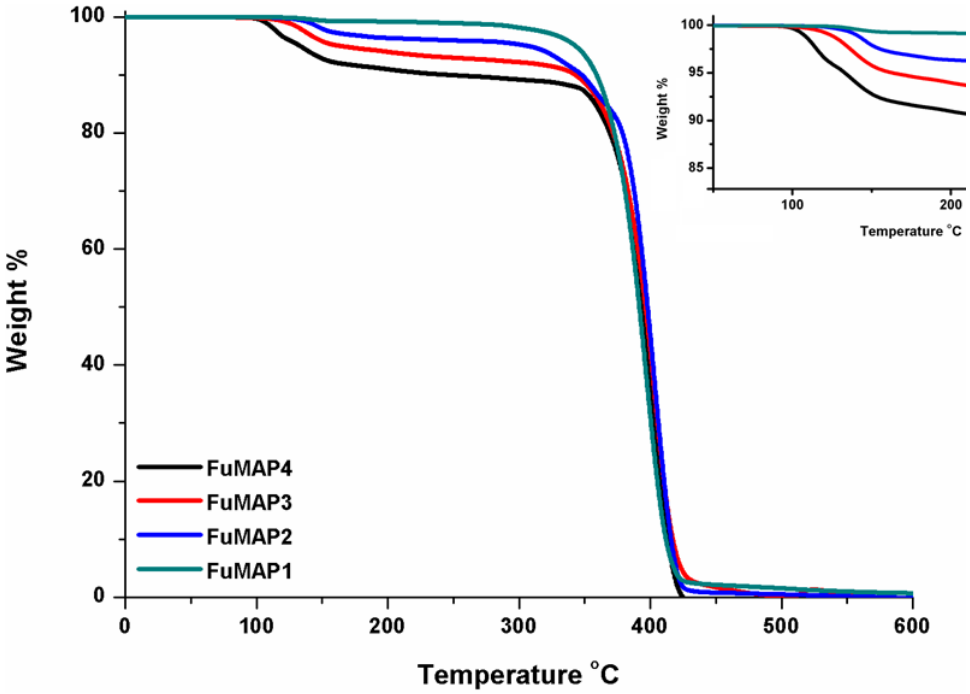


Figure 7.14. Thermogravimetric analysis plots of copolymers FuMA (P1-P4).

7.3.2. Surface Functionalization Using Catechol Chain-end Maleimide-Functionalized Copolymers

The hydrophilic copolymers containing catechol functional group at the chain end were used to fabricate water dispersible magnetic nanoparticles (MNPs) containing thiol-reactive polymeric coating. Oleic-acid coated Fe_3O_4 nanoparticles were synthesized using coprecipitation method by treatment of $FeCl_2$ and $FeCl_3$ under basic conditions using a literature protocol [190]. Nanoparticles dispersible in organic solvents such as hexane and THF were obtained and characterized for their size using TEM and DLS with diameters of 26 nm and 38 nm respectively (Figures 7.15 and 7.16). Slightly higher values were

obtained using the DLS is as expected due to consideration of the solvated ligand shell in the latter analysis.

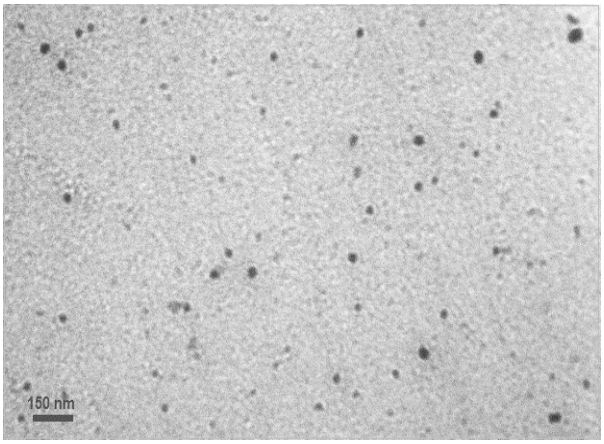


Figure 7.15. TEM image of oleic-acid coated MNPs in hexane dispersion (Scale bar: 150 nm).

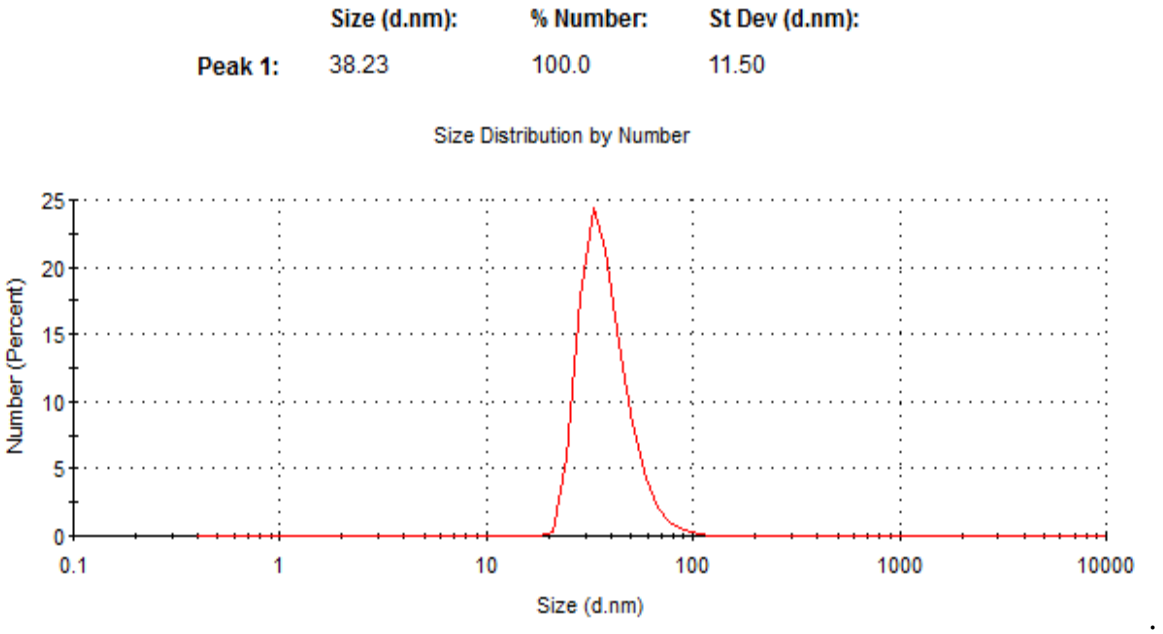


Figure 7.16. DLS analysis of oleic-acid coated MNPs in hexane dispersion.

Oleic-acid coated nanoparticles were reacted with excess amounts of catechol chain-end functionalized polymers using place-exchange reaction at 40 ^{o}C in chloroform (Figure 7.17a). After the place exchange reaction, the polymer coated nanoparticles were purified from residual unbound polymers by repeated dispersion and separation of the nanoparticles using a magnet. Due to the nature of RAFT process, some of the AIBN-initiated polymers that present in final polymer were also considered to unbound to nanoparticles and were

got rid of during purification. In contrast to the oleic acid coated nanoparticles that were readily dispersible in the hexane phase and completely non-dispersible in aqueous phase, the polymer coated nanoparticles were readily dispersible in the aqueous phase and stayed as stable suspensions over a period of few weeks (Figure 7.17b, Vials a and b). As a control experiment, place exchange reaction was carried out with a copolymer devoid of the catechol functional group at the chain end (polymer CP1, Table 7.1 Item 5) and the resultant mixture was dispersed in a hexane water biphasic solvent system. All of the nanoparticles stayed in the hexane phase, thus demonstrating the crucial role played by the catechol group in the ability to coat the nanoparticle with the hydrophilic copolymer (Figure 7.17b, Vial c).

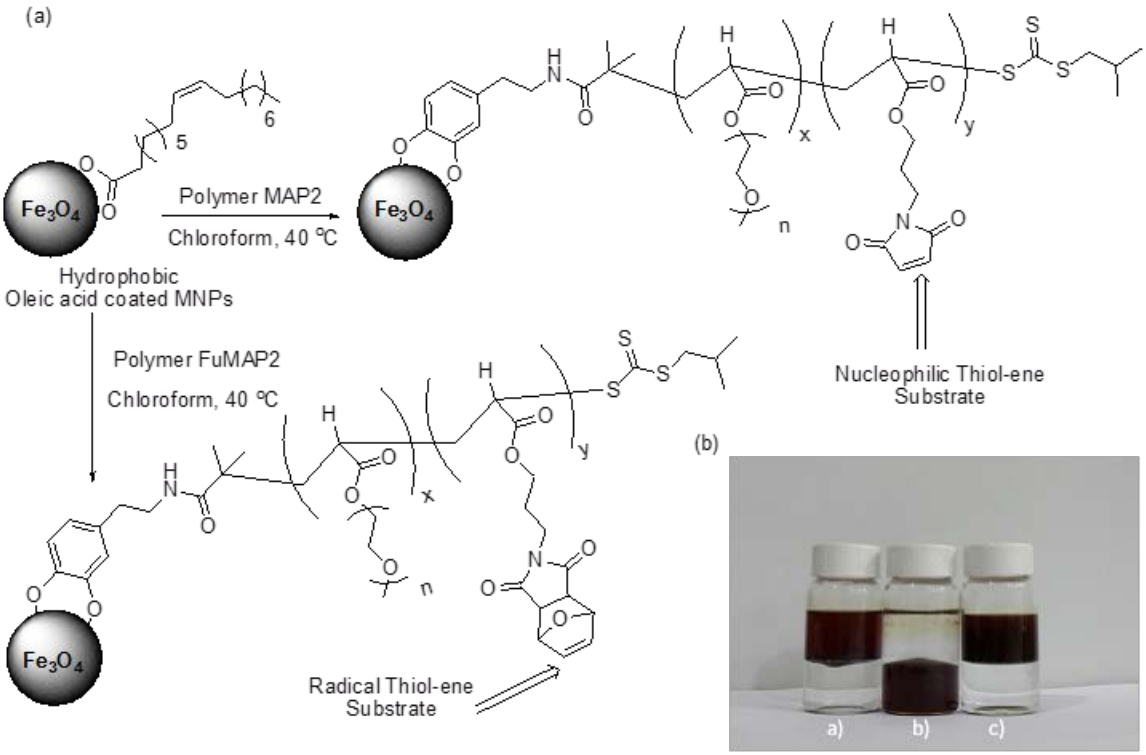


Figure 7.17. a) Surface modification of nanoparticles with hydrophilic copolymers b) The phase transfer of hydrophobic nanoparticles from hexane (upper) to aqueous (lower) phase after anchoring of catechol chain-end functionalized polymer. Vials a, b and c contain oleic acid coated MNPs, FuMAP2 copolymer coated MNPs and control MNPs.

Size analysis using TEM revealed that the individual particle size remained similar to that before modification and clustering of particles was observed in TEM due to solvent

drying effects (Figure 7.18). Size analysis of particles dispersed in THF and aqueous phase using DLS revealed diameters of 49 and 52 nm respectively (Figure 7.19).

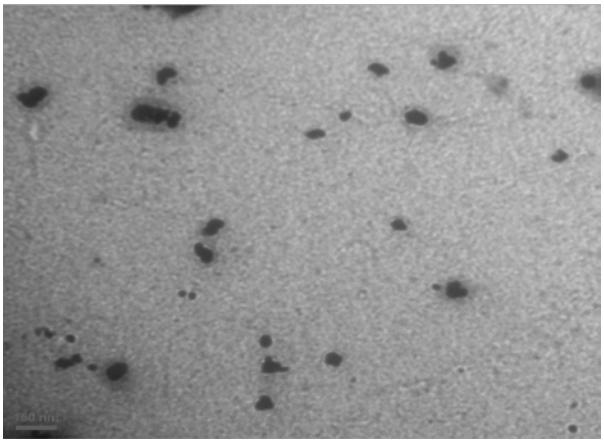


Figure 7.18. TEM image of PEGMEA/FuMA coated MNPs (Scale bar: 150 nm).

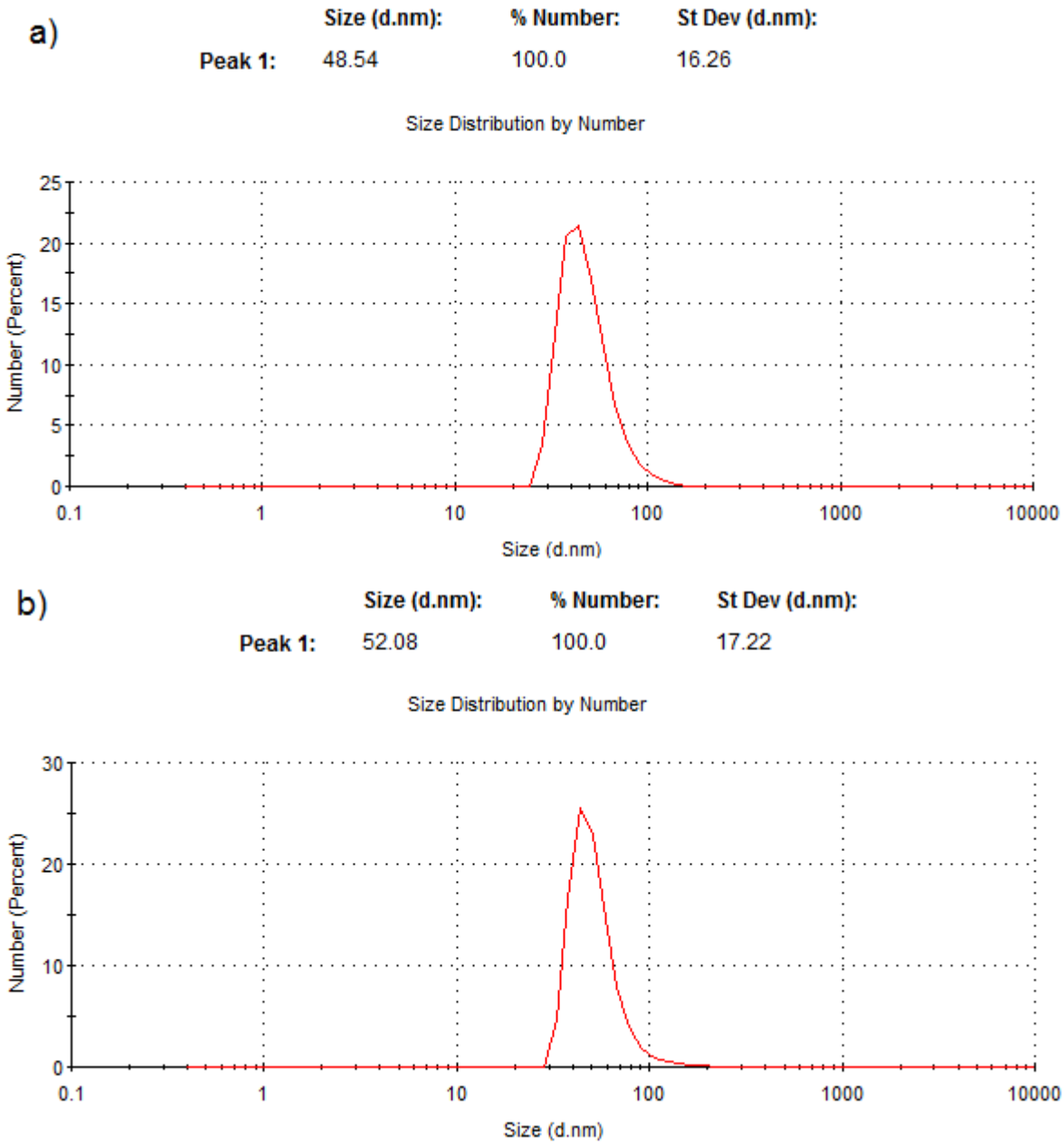


Figure 7.19. DLS analysis of polymer coated MNPs in (a) THF and (b) aqueous dispersions.

Further compositional analysis of the polymeric coating on the nanoparticle was carried out using FT-IR, UV-Vis, and TGA. The presence of the polymer coating on the nanoparticles was clearly evident from the comparison of FT-IR spectra of oleic acid coated nanoparticles with that of the polymer coated nanoparticles. The polymer coated nanoparticles exhibited all the IR absorbance peaks characteristic to the free polymers such as strong carbonyl stretching at 1730 cm^{-1} and 1702 cm^{-1} due to PEGMEA side-chain and the maleimide functional group respectively (Figure 7.20a). As expected, TGA analysis

showed higher weight loss for the polymer conjugated nanoparticles when compared to the parent oleic acid coated nanoparticles (Figure 7.20b). The marginal increase in the weight loss (15 %) can be anticipated since grafting-to techniques usually provide limited attachment of polymers due to their steric hindrance. On the basis of weight loss from TGA analysis, the polymer grafting density of nanoparticles was calculated as 0.393 nm^{-2} (Calculation of grafting density is given in the Supporting Information). Importantly, facile and stable dispersion of these polymer coated nanoparticles in aqueous phase indicates achievement of sufficient surface modification with these anchorable polymers.

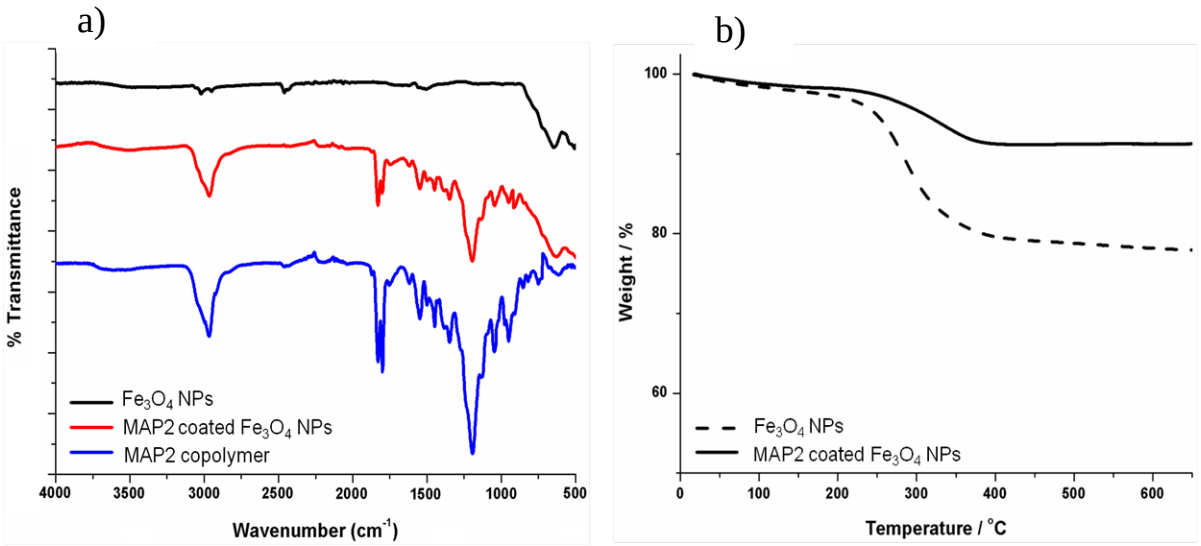


Figure 7.20. a) FT-R analysis of parent oleic acid modified nanoparticles, copolymer and copolymer coated nanoparticles, b) Thermogravimetric analysis of weight loss comparisons between parent oleic acid modified nanoparticles and polymer coated nanoparticles.

Functionalization of the modified nanoparticles was evaluated through covalent attachment of a thiol bearing hydrophobic fluorescent dye BODIPY-SH. In the first approach, nanoparticles were treated with the dye in the presence of a photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DMPA) in THF for 30 min under UV illumination (λ_{ex} = 365 nm) (Figure 7.21). Thereafter, the resulting nanoparticles were collected *via* centrifugation. These nanoparticles were redispersed in THF and collected using centrifugation to ensure that no residual unbound dye is present. It was gratifying to observe that these dye appended nanoparticles were readily dispersible in water and were fluorescent under UV illumination. No physisorbed hydrophobic dye was present since

treatment of the nanoparticles aqueous dispersion with an organic solvent did not show any transfer of hydrophobic dye to the organic phase. As a control experiment, nanoparticles coated with catechol chain end functional polymer devoid of the FuMa monomer (polymer CP2, Table 7.1 Item 6) were treated with the thiol containing dye under identical conditions. No attachment of dye to these hydrophilic polymer coated nanoparticles was inferred since their aqueous dispersion was non-fluorescent.

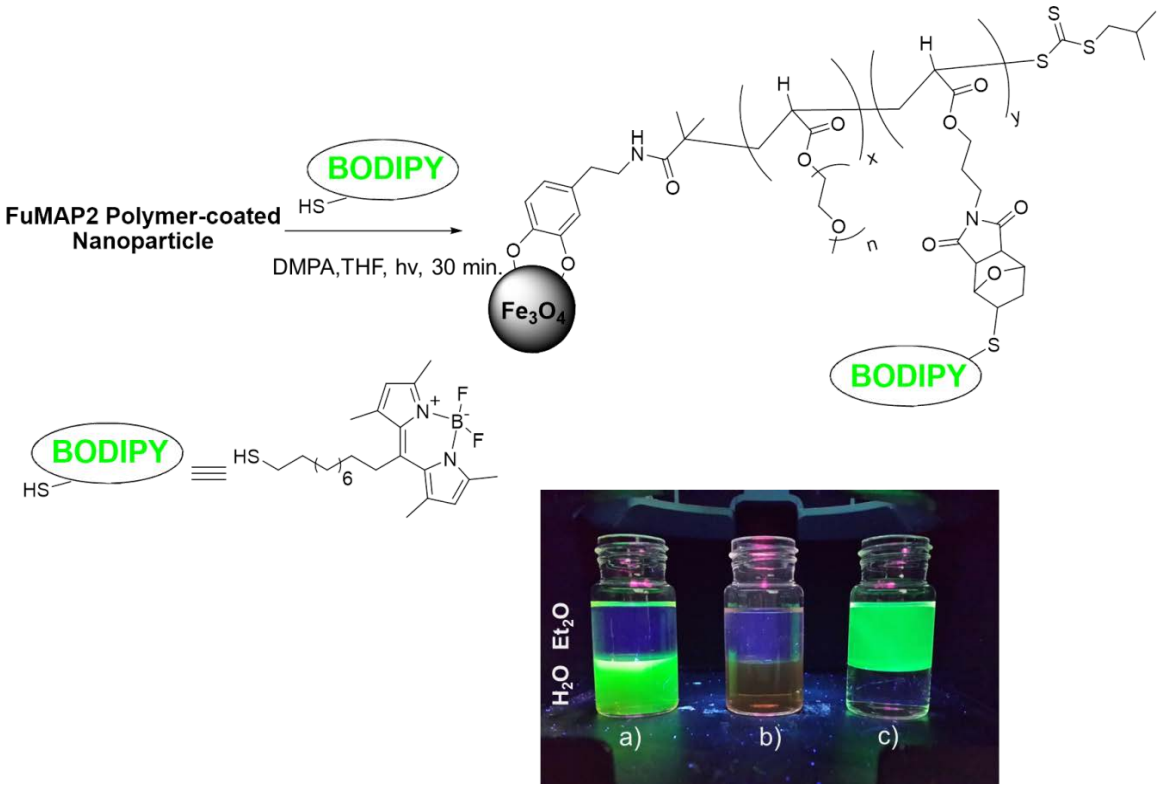


Figure 7.21. Thiol-conjugation onto FuMAP2-copolymer coated nanoparticles using radical thiol-ene reaction. a) FuMAP2-copolymer coated nanoparticles after dye conjugation, b) Control nanoparticles coated with CP2 polymer, c) Free dye. The upper phase is ether and lower phase is water. The photograph was taken under UV illumination at 365 nm.

Although the nanoparticles containing the furan-protected maleimide units were readily functionalizable via photochemical radical thiol-ene reactions, we also investigated the nanoparticles coated with polymers containing reactive maleimide groups towards conjugation. This strategy provides an attractive alternative since it can be accomplished under reagent free conditions without the need of UV irradiation that can lead to

photochemically induced damage for certain molecules. Polymer coated nanoparticles were reacted with BODIPY-SH in THF at 40 ^{o}C without addition of any other reagents (Figure 7.22). After thorough purification to remove any unreacted dye molecules, these nanoparticles were dispersed in water. The obtained aqueous dispersion of the nanoparticles was fluorescent under UV illumination. This demonstrates that successful conjugation is realized since the dye is insoluble in the aqueous phase and prefers the organic phase. As a control experiment, nanoparticles coated with CP2 polymers devoid of maleimide side chains subjected to similar conjugation reaction did not show any fluorescence.

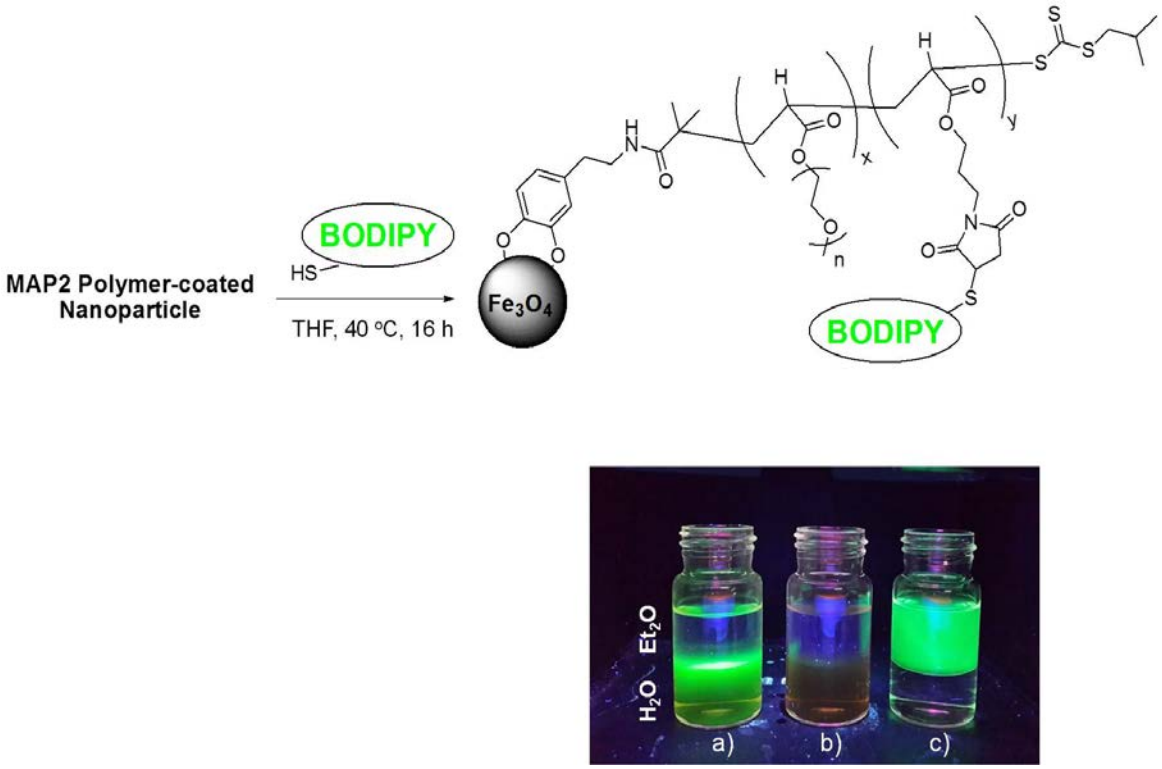


Figure 7.22. Thiol-conjugation onto MAP2-copolymer coated nanoparticles using nucleophilic thiol-ene reaction a) MAP2-copolymer coated nanoparticles after dye conjugation, b) Control nanoparticles coated with CP2 polymer c) Free dye. The photograph was taken under UV illumination (λ_{ex} = 365 nm).

7.4. Conclusion

Polymers containing maleimide based thiol-reactive side chains that contain a catechol group at their chain end were synthesized using a Diels-Alder/retro Diels-Alder reaction sequence. Polymers with varying amounts of the maleimide group can be prepared with good control over molecular weight and narrow distribution using RAFT polymerization. The catechol functional group at polymer chain end allows the fabrication of water dispersible magnetic nanoparticles that can be subsequently functionalized by thiol bearing molecules. Thiol conjugation onto polymer coated nanoparticles could be achieved using either photo-initiated radical thiol-ene or through nucleophilic Michael addition reactions. Attachment of a hydrophobic fluorescent dye was accomplished on polymer coated nanoparticles using these methods to obtain hydrophilic fluorescent magnetic nanoparticles. One can envision that these thiol-reactive water soluble surface-anchorable polymers will find applications in many areas of biomedical sciences that utilize fabrication of functional coatings.

8. DESIGN AND SYNTHESIS OF MULTIFUNCTIONAL POLYMERS FOR POTENTIAL TARGETED DRUG DELIVERY APPLICATIONS

8.1. Introduction

Development of new drug delivery systems for the treatment of several diseases is a central research focus that many researchers from both academy and industry are currently investigating. Drug delivery systems (DDSs) employ the methods, approaches and technologies to transport a pharmaceutical agent to specific parts of the body, in humans or animals, to safely achieve the therapeutic effect of medications. DDSs modify the release profiles of the drugs as well as adjust the absorption, distribution, metabolism and elimination of pharmaceutical agents to improve their efficacy.

The capability of delivering effective dosages of medications to specific sites of body that DDSs have indeed originates from two distinct concepts: targeted drug delivery and sustained/controlled drug delivery. Targeted drug delivery systems are DDSs in which the medication is delivered to patient in a way of increasing the drug concentration in some body parts, organs or tissues relative to the others. The aim in a targeted drug delivery is mainly to localize the therapeutic agent in a diseased area of body and prolong the drug/tissue interaction. Localization of the drugs in diseased tissues increases the efficacy of treatment as well prevents healthy tissues from damages of toxic drug compounds. The other concept, sustained or controlled drug delivery aims gradual release of the drugs from transporting delivery systems in order to maintenance of drugs concentrations at therapeutically desirable levels to avoid under or overdosing.

A wide range of DDSs including liposomes, nanoparticle-based carriers, lipoprotein-delivery systems and dendrimers are utilized as delivery vehicles. Drug delivery systems utilizing natural or synthetic polymers is a growing field in which micellar, composite polymeric carriers or linear polymers with covalently or non-covalently attached drug payloads have become a new domain in development of DDSs for the treatment of numerous diseases. Polymer-based DDSs include protein–polymer conjugates, hydrogels, microspheres, nanoparticles and polymer-drug conjugates [191] (Figure 8.1). These polymeric drug carriers now become indispensable delivery systems and referred as

polymer therapeutics or nanomedicines that find applications especially in treatment of cancer related diseases.

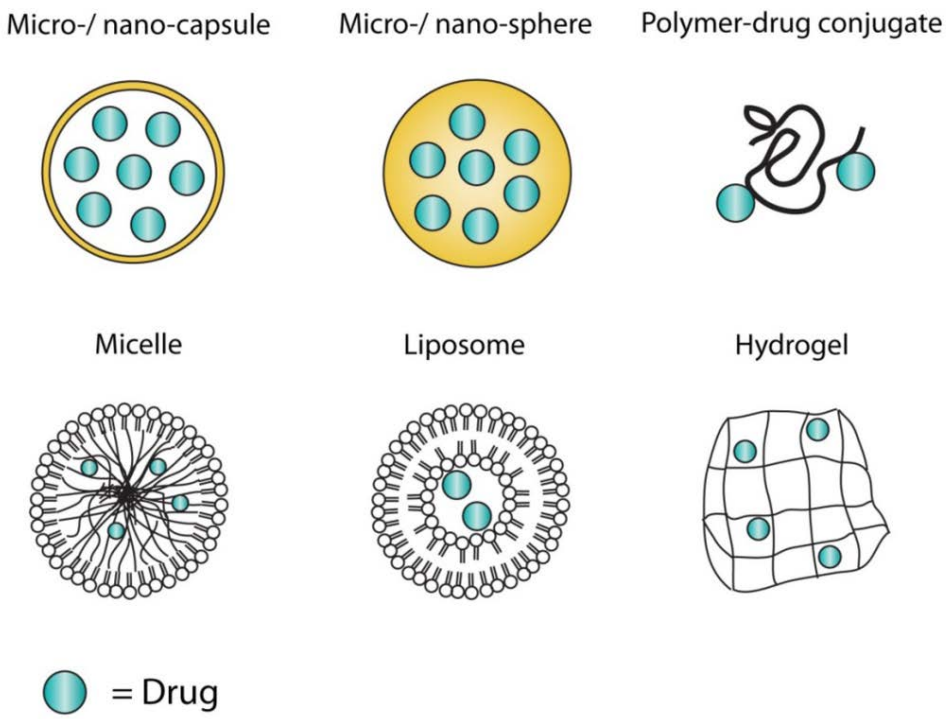


Figure 8.1. Various polymer based materials with different structures and morphologies used as drug delivery systems (From [192]).

Polymer drug conjugates enrolls a vital role in development of new methods in cancer diagnosis and treatment. In these macromolecular constructs, drug molecules are generally attached to polymer chain via covalent bonding, making the active drugs prodrugs. Prodrugs are inactive forms of drugs that they remain intact during the delivery to the action center. When the polymer drug conjugate reaches the site of action, prodrug is activated by action of specific conditions. The reconversion of active drugs may be accounted by cleaving a bond, an enzymatic action or any other trigger. Attaching the drug molecules to polymers are usually accomplished by using linkers. Upon the action of specific conditions on the linkers, the drug molecules are released to the medium.

The linker choice to construct the polymer drug conjugate is crucial to achieve the desired action of drug delivery system. Commonly employed linkers include biodegradable linkers, hydrolytically cleavable linkers, enzymatically cleavable linkers, pH or light sensitive linkers and stimuli responsive linkers. Most of the linkers are based on reversibly

cleavable covalent bonds that are designed to degrade in different conditions to release the drug molecules (Figure 8.2). This type of drug release can be considered as a controlled release as the drug release is governed by the triggering conditions. To utilize the polymer drug conjugates in targeted drug delivery, targeting molecules can also be conjugated to the construct. With the help of targeting moieties on the delivery system, specific delivery to desired site of body could be maintained.

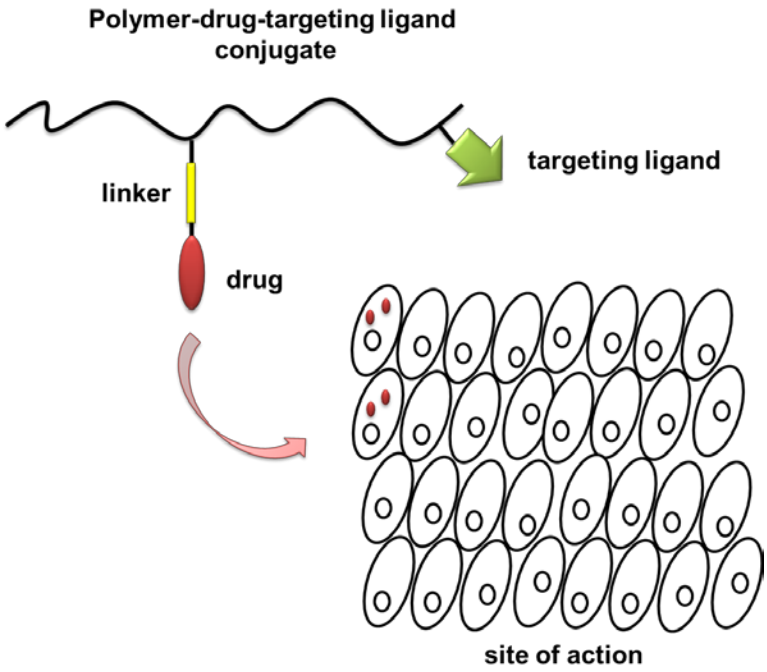


Figure 8.2. Rationale for drug delivery via polymer-drug conjugates.

Activation of prodrugs may involve the hydrolytic cleavage of chemical linkages. In this approach, the drug is attached to the polymer conjugate via hydrolytically cleavable covalent bonds and the drug is released by the degradation of the linker structure. This strategy allows the controlled and prolonged drug release in which the release kinetics are controlled by changing certain temperature and pH conditions. A variety of chemical linkages including esters, imines, carbonates, carbamates and hydrazones are employed as hydrolytically cleavable groups (Figure 8.3).

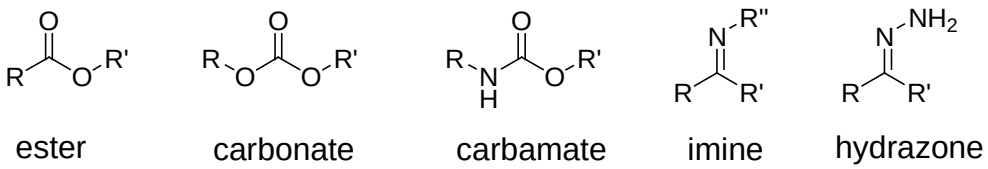


Figure 8.3. Commonly employed hydrolytically cleavable linkages for attachment of therapeutic agents to polymers.

The design of hydrolytically cleavable linkers has utmost importance to control the release rates of the drugs in body. In many studies, it was demonstrated that prodrugs containing ester linkages show fast hydrolysis whereas carbamate based linkers show higher stability in plasma [193]. In ideal drug polymer conjugates, linkers are required to be stable in plasma while showing inherent biodegradability in diseased tissues. Carbamate based linkers thus attracted considerable attention based on the plasma stability and cleavage conditions [190–194]. We have realized that polymer-drug conjugates bearing drug molecules covalently bonded via carbamates linkages may possess important advantages in controlled drug release applications. Due to the relatively slow hydrolysis property of carbamates, conjugates bearing carbamate-linked drug molecules may provide different release profiles than corresponding esters, carbonates or amides.

In this study, we report on the synthesis of novel polymers containing activated carbonate groups at side chains and demonstrate their application towards functionalization with an amine containing drug molecule to prepare polymer drug conjugates. Poly(oligo(ethylene glycol) methacrylate) (PEGMEMA)-based hydrophilic polymers incorporating NHS-activated carbonate group containing side-chains were synthesized using reversible addition fragmentation chain transfer (RAFT) polymerization. A furan-protected maleimide-containing chain transfer agent was also synthesized and used in the copolymerization of monomers. Removal of the furan protecting group after polymerization yields end chain thiol-reactive polymers possessing intact activated carbonate units at their side chains. Polymers with varying composition of the NHS-activated carbonate group containing monomer were obtained with narrow molecular weight distributions as inferred from size exclusion chromatography. Obtained polymers were characterized using proton nuclear magnetic resonance (^{1}H NMR) to establish their chemical composition. The obtained maleimide end functional and activated carbonate side chain functional polymers were evaluated in functionalization with thiol-bearing and

amine-bearing bio-related compounds. Side chain activated carbonate groups were functionalized with an amine containing anticancer drug doxorubicin. The end chain maleimide group is also functionalized with folic acid and cyclic RGD peptide molecules through thiol-maleimide reaction. Folic acid and cyclic RGD molecules were installed considering their targeting group properties in drug delivery applications. *In vitro* drug release was determined under buffer conditions. Their cytotoxicity was evaluated against SKOV-3 (human ovarian cancer) and A549 (human lung adenocarcinoma) cell lines. Internalization of the conjugates on SKOV-3 and A-549 cell lines was investigated.

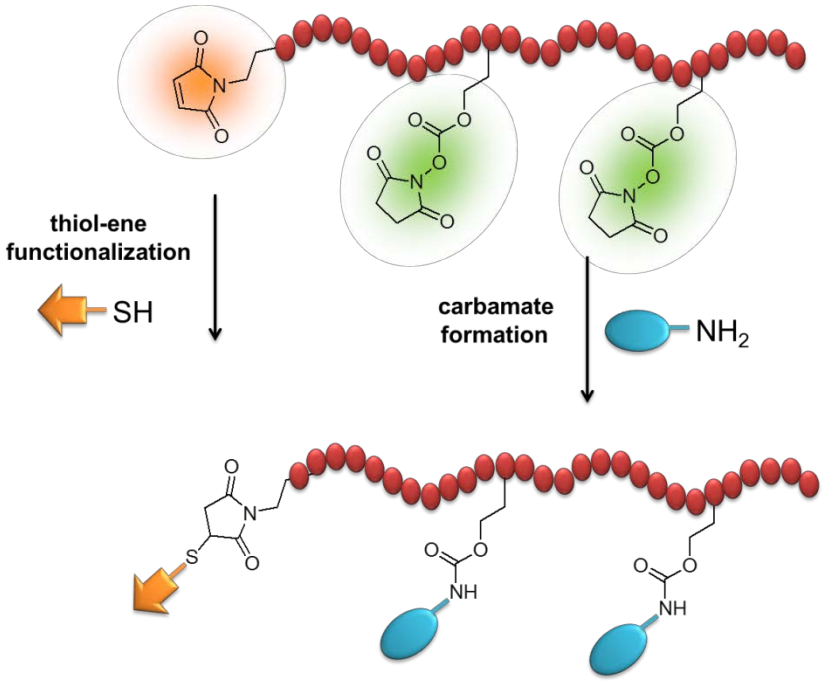


Figure 8.4. Design of thiol and amine reactive copolymer capable of sequential functionalization.

8.2. Experimental

8.2.1. Materials and Characterization

Poly(ethylene glycol) methyl ether methacrylate (PEGMEMA, M_n=300) and di(ethylene glycol) methyl ether methacrylate (DEGMA) were purchased from Sigma Aldrich and purified by passing through an activated aluminum oxide column prior to use. 2-cyano-2-propyl dodecyl trithiocarbonate, 4-cyano-4-[(dodecylsulfanylthiocarbonyl) sulfanyl]pentanoic acid, 4-dimethylaminopyridine (DMAP), triethylamine were purchased

from Sigma Aldrich and used as received. 2,2′-Azobis(2-methylpropionitrile) (AIBN) was purchased from Sigma Aldrich and recrystallized from methanol before use. 1-Ethyl-3-(3-dimethylamino propyl) carbodiimide (EDCI) was obtained from Alfa Aesar. Anhydrous sodium sulfate, dichloromethane, hexane, ethyl acetate and methanol were purchased from Merck. Furan-protected maleimide containing alcohol (FM-OH) [108], N-hydroxy succinimide-based carbonate monomer (SCEMA) [199], thiolated folic acid (FA-SH) [200] and cylic-RGD peptide (c(RGDfC)) [140] were prepared according to previously reported literature protocols. Doxorubicin hydrochloride was obtained from Sigma Aldrich. Elemental analysis data was obtained using a Thermo Electron S.p.A Flash EA® 1112 elemental analyzer. The molecular weights were determined by gel permeation chromatography analysis using Shimadzu RID-10A refractive index detector and PSS SDV Linear M column calibrated with polystyrene standards. THF was used as eluent at a flow rate of 1 mL/min at 30 ^{o}C. PSS WinGPC software was used to process data. Characterization of compounds and copolymers were performed using ^{1}H NMR, ^{13}C NMR spectroscopy (Varian 400 MHz). UV studies were performed with a Varian Cary 50 Scan UV/vis spectrophotometer.

8.2.2. Synthesis of Furan Protected Maleimide-Functionalized CTA (CTA-Mal)

In a 10 mL round-bottom flask equipped with a stir bar, a solution of 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (200.0 mg, 0.49 mmol), FM-OH (145.0 mg, 0.65 mmol), and DMAP (12.0 mg, 0.10 mmol) in 4 mL of CH_2Cl_2 was cooled to 0 °C under N_2. In another flask, EDC (104.0 mg, 0.54 mmol) was dissolved in 2 mL of CH_2Cl_2 and added dropwise to the reaction flask. The reaction mixture was stirred at 0 °C for one hour and then allowed to warm to room temperature and stirred 12 h. Then, the reaction mixture was washed with $NaHCO_3$ (sat'd), dried over Na_2SO_4, and evaporated *in vacuo* to give viscous oil. The crude product was purified by column chromatography on silica with hexane and EtOAc (1:1) affording the final product as a yellow oil (220.0 mg, 74 %). ^{1}H NMR ($CDCl_3$) δ (ppm): 6.50 (s, 2H, C*H*=C*H*); 5.26 (s, 2H, C*H* bridgehead protons); 4.06 (t, 2H, *J* = 6.6 Hz, OCH_2); 3.55 (t, 2H, *J* = 7.3 Hz, NCH_2); 3.32 (t, 2H, *J* = 6.8 Hz, SCH_2); 2.84 (s, 2H, C*H*-C*H*, bridge protons); 2.35-2.65 (m, 4H, CH_2CH_2); 1.90 - 1.96 (m, 4H, $CH_2CH_2CH_2$); 1.88 (s, 3H, CH_3); 1.69 (m, 2H); 1.39 (m, 2H); 1.26 (br s, 16 H); 0.87 (t, 2H, *J* = 6.2 Hz, CH_3). ^{13}C NMR ($CDCl_3$) δ (ppm): 212.94, 176.1, 171.3, 136.4, 119.2, 80.9, 64.8, 47.3, 46.3, 37.0, 35.6, 33.8, 31.8, 29.6, 29.5, 29.5, 29.3, 29.3, 29.0, 28.9,

27.6, 26.5, 24.8, 22.6, 14.0 Anal. Calcd. [$C_{20}H_{44}N_2O_5S_3$]: C, 59.18; H, 7.28; N, 4.60; S, 15.80. Found: C, 58.36; H, 7.59; N, 4.93; S, 15.27.

8.2.3. Homopolymerization of SCEMA via RAFT

SCEMA (270.0 mg, 1.0 mmol), CTA (8.6 mg, 0.025 mmol) and AIBN (0.40 mg, 0.0025 mmol) were dissolved in dioxane (1 mL) and placed in a sealed round-bottom flask equipped with a magnetic stir bar (Figure 8.5). The reaction mixture was purged with N_2 for 15 min and stirred at 70 °C for 12 h. After polymerization, the solvent was removed under vacuo and the residue was re-dissolved in dichloromethane (1 mL) and precipitated into the cold Et_2O. The precipitated polymer was dried under vacuo and characterized using SEC (Figure 8.6) and ^{1}H NMR.

8.2.4. Copolymerization of SCEMA with OEGMA Monomers via RAFT

Polymerization procedure for copolymers P1 and P3: OEGMA (DEGMA, 188.0 mg, 1.0 mmol for P1 and PEGMEMA, 300.0 mg, 1.0 mmol for P2), SCEMA (135.0 mg, 0.50 mmol), CTA (13.0 mg, 0.0375 mmol) and AIBN (0.60 mg, 0.00375 mmol) were dissolved in dioxane (1.50 mL) and placed in a sealed round-bottom flask equipped with a magnetic stir bar. The reaction mixture was purged with N_2 for 15 min and stirred at 70 °C for 12 h. After polymerization, the solvent was removed under vacuo and the residue was re-dissolved in dichloromethane (1 mL) and precipitated in cold Et_2O. The precipitated polymer was dried under vacuo and characterized using SEC (Figure 8.6) and ^{1}H NMR (Figure 8.7).

8.2.5. Determination of Reactivity Ratios in SCEMA/DEGMA Copolymers

For the determination of reactivity ratios of SCEMA and DEGMA monomers, a series of copolymers with different feed ratios under the same conditions as above were prepared, but reaction times were restricted to low conversions of comonomers (<10 %). The copolymerization reactions were performed using [SCEMA]:[DEGMA] concentrations of 2:1, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7 and 1:8. The polymerization reactions were terminated by rapid cooling of reaction mixtures and the products were isolated by precipitation in cold Et_2O. The copolymer compositions were determined by ^{1}H NMR.

8.2.6. Copolymerization of SCEMA and PEGMEMA Monomers with CTA-Mal via RAFT

PEGMEMA (540.0 mg, 1.80 mmol), SCEMA monomer (54.0 mg, 0.20 mmol), CTA-Mal (15.0 mg, 0.025 mmol) and AIBN (0.40 mg, 0.0025 mmol) were placed in a sealed tube equipped with a magnetic stir bar. The mixture was dissolved in dioxane (2 mL) and purged with N_2 for 15 min. The reaction mixture was stirred at 70 °C for 12 h. After polymerization, the solvent was removed under vacuo and the residue was re-dissolved in dichloromethane (1 mL) before precipitating in cold Et_2O. The precipitated polymer was dried under vacuum and characterized using SEC (Table 8.2 and Figure A.10) and ^{1}H NMR (Figure 8.10).

8.2.7. Radical Cross-Coupling of Trithiocarbonyl End-Functional Polymer with AIBN

Copolymer FM-P (200.0 mg, 0.0093 mmol) and AIBN (40.0 mg, 0.23 mmol) were dissolved in DMF (4 mL) in a sealed tube. The reaction mixture was purged with nitrogen for 15 minutes and stirred at 70 °C for 4 h. After the reaction, the solvent was removed *in vacuo* and the resulting polymer was purified by precipitating in the cold Et_2O.

8.2.8. Post-polymerization Activation of Maleimide Functional Group

The copolymer (200 mg) was dissolved in anhydrous toluene (150 mL) and refluxed under N_2 atmosphere. After 8 h, the solution was cooled to room temperature and the solvent was evaporated under vacuo to yield copolymer M-P in quantitative yield. Thus obtained polymer was characterized using SEC (Figure A.10) and ^{1}H NMR (Figure 8.12).

8.2.9. Functionalization of Polymer M-P with Doxorubicin Conjugation

Copolymer M-P (200.0 mg, 0.010 mmol) was dissolved in dichloromethane (5 mL). Doxorubicin hydrochloride (32.0 mg, 0.054 mmol) and triethylamine (9.0 µL, 0.064 mmol) was then added to this solution and the reaction mixture was stirred at 0 ^{o}C for 2 h. After the reaction, the purified polymer M-P-Dox was obtained by dialysis against acetonitrile (MWCO 3,500).

8.2.10. Functionalization of Maleimide-End Functional Polymer M-P-Dox with Thiol-Functionalized Folic Acid and cRGD

Polymer FA-P-Dox: M-P-Dox (100.0 mg, 0.0042 mmol) was dissolved in DMSO (0.6 mL) and degassed for 10 minutes. Folic acid thiol (FA-SH) (2.6 mg, 0.005 mmol) and triethylamine (0.1 eq. per thiols) was then added to this solution. The reaction mixture was stirred at room temperature for 12 h and the resulting polymer was purified by dialysis against DMSO and acetonitrile (MWCO 3,500) to obtain functionalized polymer FA-P-Dox.

Polymer R-P-Dox: M-P-Dox (100.0 mg, 0.0042 mmol) was dissolved in DMSO (0.6 mL) and degassed for 10 minutes. c(RGDfC)-thiol (2.9 mg, 0.005 mmol) and triethylamine (0.1 eq. per thiols) was then added to this solution. The reaction mixture was stirred at room temperature for 12 h and the resulting polymer was purified by dialysis against DMSO and acetonitrile to obtain functionalized polymer R-P-Dox.

8.2.11. *In Vitro* Drug Release Experiments

Doxorubicin conjugated polymers (FA-P-Dox and R-P-Dox, 10 mg each) with either folate thiol or c(RGDfC)-thiol end groups were dissolved in acetate buffer (pH 5.0) and phosphate buffered saline (PBS, pH 7.4) solutions (2 mL), sealed in a dialysis bag (MWCO: 3.5 kDa). The dialysis bags were incubated in a 15 mL release medium at 37 °C under 100 rpm oscillation. The solution outside the dialysis bag was removed from the release medium within predetermined time intervals and replaced with same volume of fresh buffer. The amount of drug in collected media was determined spectrophotometrically using the absorbance of doxorubicin at 488 nm with the help of a calibration curve. The results were expressed in terms of cumulative release as a function of time.

8.3. Results and Discussion

8.3.1. Copolymerization of NHS-activated Carbonate Group Containing Monomer (SCEMA) with OEGMA Monomers

RAFT copolymerization of NHS-activated carbonate group containing monomer (SCEMA) with hydrophilic oligo (ethylene glycol) based monomers DEGMA and PEGMEMA were investigated. We recently demonstrated the synthesis and AIBN initiated free radical polymerization of this novel monomer with various other monomers [199]. This monomer was utilized as an amine reactive substrate to prepare side chain functional polymers. The eventual formation of carbamate linkages via the functionalization of these activated carbonate groups with amine containing molecules would be considered as a convenient method to obtain polymers with side chain hydrolysable carbamate groups. In this study, utilization of this monomer for the preparation of water soluble, functionalizable polymers via controlled radical polymerization was demonstrated.

RAFT homopolymerization and copolymerization of SCEMA with hydrophilic monomers DEGMA and PEGMEMA in the presence of a trithiocarbonyl chain transfer agent (CTA) was investigated (Figure 8.5). The polymers P1-P5 with different SCEMA feed ratios were synthesized in dioxane at 70 ^{o}C (Table 8.1, Items 1-5). Polymers were obtained in pure form after removal of unreacted monomers upon their precipitation with Et_2O. Polymers were obtained with relatively high conversion in dioxane; polymerization reactions conducted in DMF provided similar polymer conversions as well. According to SEC analysis, polymers were obtained with good control over molecular weight and narrow polydispersities were observed (Table 8.1 and Figure 8.6).

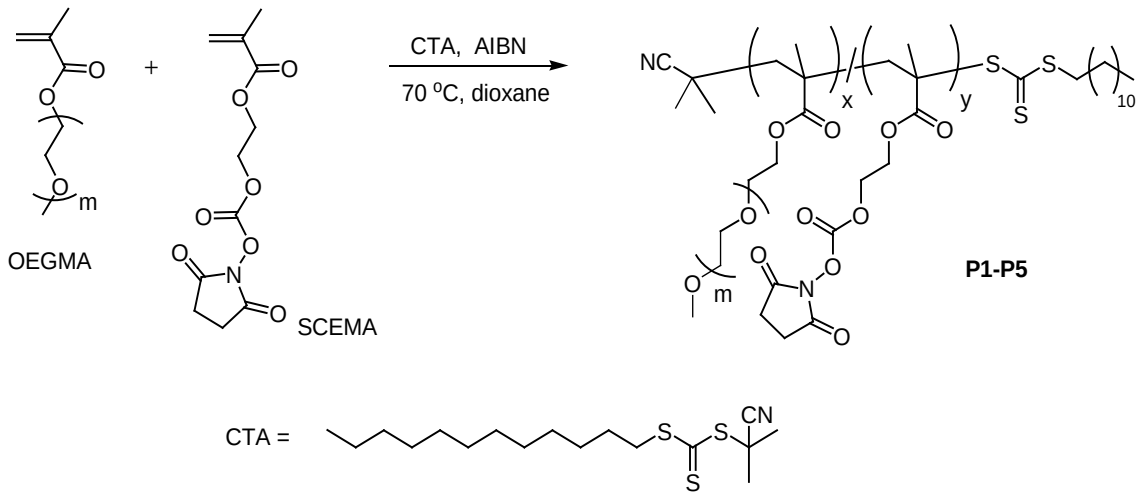


Figure 8.5. Synthesis of the RAFT copolymers containing reactive carbonate side chains.

Table 8.1. Conversion, molar mass, and polydispersity data for the RAFT mediated polymers.

Item	Polymer[a]	SCEMA : OEGMA		Conv. %	$M_{n\ (theo)}$	$M_{n,GPC}$[c]	M_w/M_n[c]
		Theoretical	Observed[b]				
1	P1	1 : 0	--	71	8050	10100	1.31
2	P2	1 : 2	1 : 2.76	74	6735	8300	1.26
3	P3	1 : 4	1 : 5.66	83	7140	8700	1.24
4	P4	1 : 2	1 : 2.43	86	10330	12600	1.22
5	P5	1 : 4	1 : 5.32	77	9400	11250	1.23

[a] $[M]_o:[CTA]_o$ = 40, 70 °C, 12 h, solvent dioxane. OEGMA: For P2 and P3, DEGMA and for P4 and P5, PEGMEMA. [b] Determined by ^{1}H NMR. [c] Estimated by SEC eluted with THF using polystyrene standards.

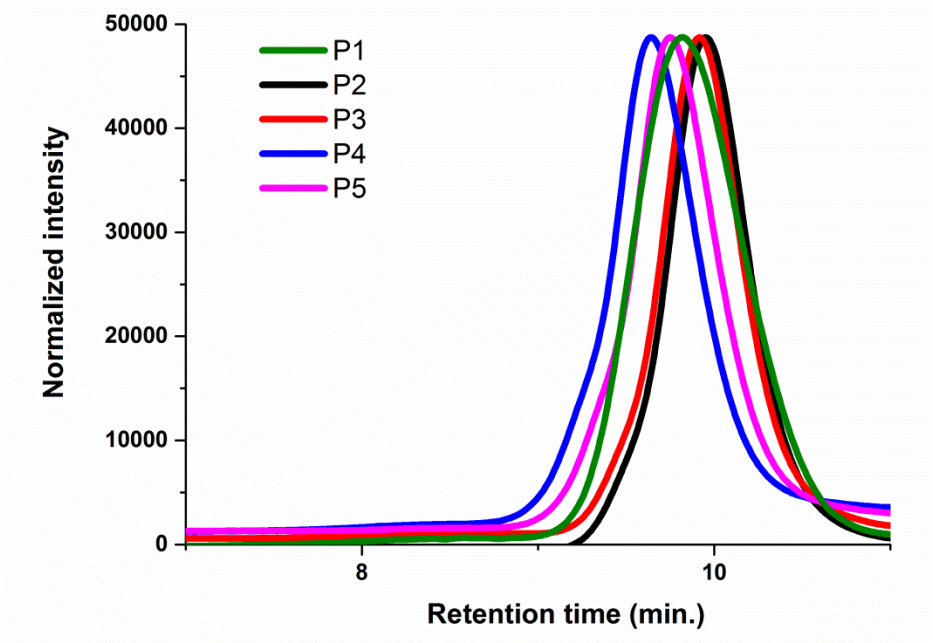


Figure 8.6. SEC traces of RAFT copolymers containing reactive carbonate side chains.

Analysis of these copolymers using ^{1}H NMR revealed successful incorporation of the SCEMA monomer into the copolymers, as was evident from the proton resonances around 6.51, 5.23 and 4.54 and 2.86 ppm due to the carbonate methylene and succinimide protons, respectively (Figure 8.6). The extent of incorporation of the monomers into the final copolymers for particular feed ratios were obtained by comparison of integration of the proton resonance at 3.38 ppm due to the terminal methyl ether group of the OEGMA monomer with the succinimidyl protons of SCEMA at 2.86 ppm. The experimentally obtained monomer compositions of the copolymers show that the incorporation of the carbonate monomer is lower than aimed in the feed (Table 8.1, Items 2-5). This discrepancy can be attributed to the steric hindrance of the pendant succinimidyl group in the monomer. We envisioned that the determination of reactivity ratio of SCEMA monomer under RAFT conditions would provide a better control on designed copolymer compositions. Thus, a linear least-squares regression analysis was used to determine the reactivity ratio of SCEMA to DEGMA monomer. By exploiting the conventional linearization method of Kelen-Tudos (K-T) [201], the reactivity ratios of SCEMA and DEGMA were found 0.72 and 1.24, respectively. Therefore, the sequence of the copolymer structure was found to be statistical in structure with less SCEMA units than the aimed feed ratio.

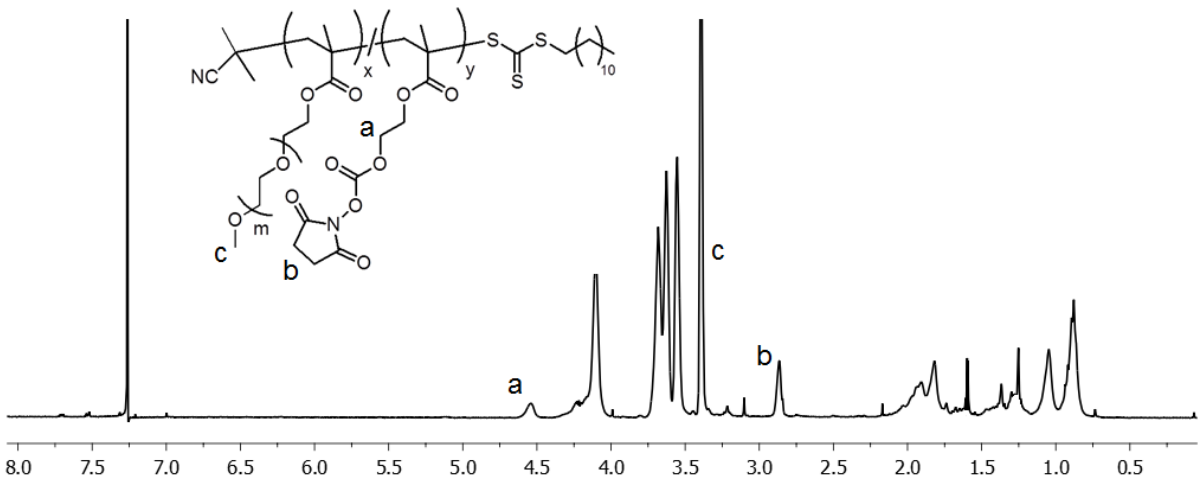


Figure 8.7. ^{1}H NMR spectrum of SCEMA/DEGMA copolymer P3 in $CDCl_3$.

8.3.2. Synthesis of Maleimide End Functional, Side Chain NHS-activated Carbonate Group Containing Copolymer

After the brief survey of RAFT polymerization of SCEMA monomer with hydrophilic DEGMA and PEGMEMA monomers, a novel furan-protected maleimide containing CTA was utilized in the RAFT polymerization of SCEMA with PEGMEMA monomer. The functional group containing CTA was aimed to use in preparation of orthogonally functionalizable copolymer of SCEMA and PEGMEMA that contains both amine reactive and thiol reactive groups allowing the sequential post-polymerization modification of copolymer. Thus obtained functional hydrophilic copolymer with side chain activated carbonate groups and end chain maleimide group has been considered as a versatile platform for preparation polymer-drug conjugates by attachment of target molecules.

A novel furan-protected maleimide functional group containing CTA (CTA-Mal) was synthesized for the synthesis of maleimide end functionalized copolymers. CTA-Mal chain transfer agent was synthesized by treatment of a furan-protected maleimide containing alcohol with 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid in EDC and DMAP mediated esterification reaction (Figure 8.8).

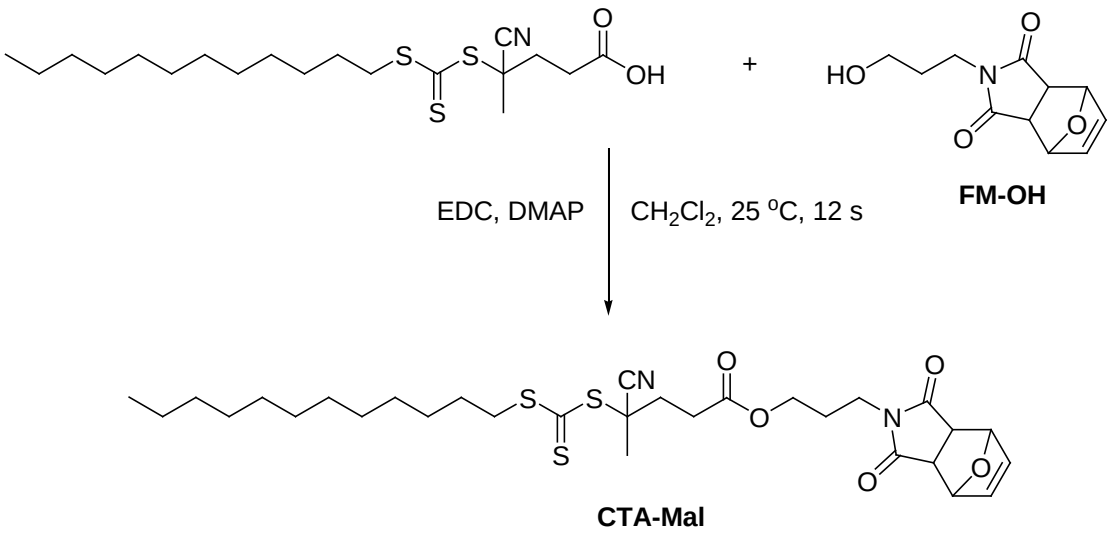


Figure 8.8. Synthesis of furan-protected maleimide functional group containing CTA-Mal.

The copolymer FM-P with a (1:9) SCEMA/PEGMEMA content was synthesized by copolymerization of two monomers in the presence of furan-protected maleimide containing CTA-Mal in dioxane at 70 °C (Figure 8.7, Table 8.2). The relatively low ratio between [CTA]/[AIBN] (10/1) was considered to be moderate to reduce the amount of polymer derived from the initiator still allowing the control on polymerization. During the polymerization, the temperature was kept low enough to avoid any retro Diels-Alder reaction mediated deprotection of the masked maleimide group yet high enough to obtain polymer with intended molecular weight within reasonable reaction time.

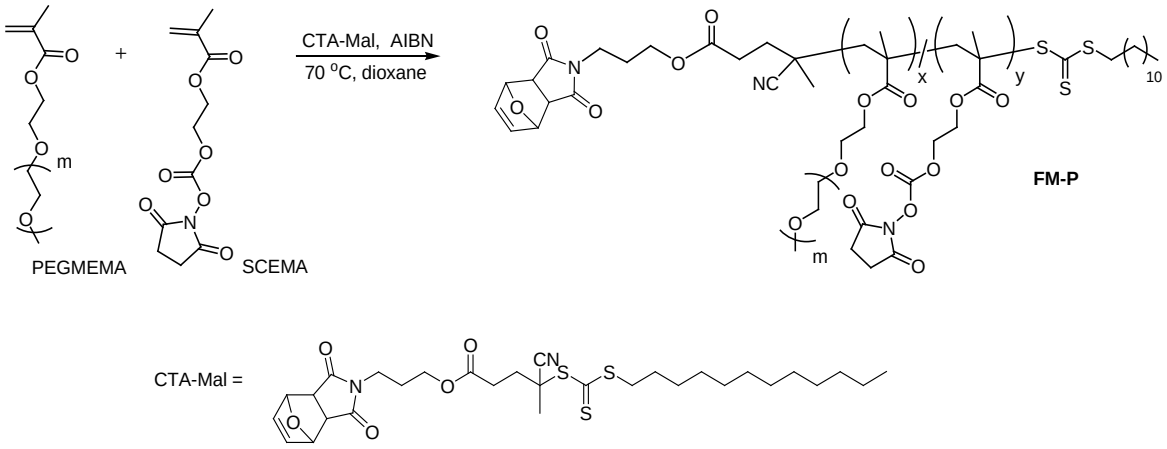


Figure 8.9. Synthesis of the copolymer FM-P containing reactive carbonate side chains and protected maleimide end group.

Table 8.2. Polymerization Conditions and Copolymer Characterizations of FM-P.

Polymer[a]	$F_{theo.}$[b] **(%)**	$F_{calc.}$[c] **(%)**	**Conv. (%)**	$M_{n,theo.}$ **(g/mol)**	$M_{n,NMR}$[c] **(g/mol)**	$M_{n,SEC}$[d] **(g/mol)**	M_w/M_n[d] **(g/mol)**
FM-P	10 : 90	7.1 : 92.9	76	18700	21500	14800	1.32

[a] $[M]_o/[CTA]_o$: 80; 70 °C; 12 h; solvent dioxane. [b] $F_{theo.:}$ [SCEMA]:[PEGMEMA]; [c] Determined by ^{1}H NMR.
[d] Estimated by SEC eluted with THF, using polystyrene standards.

Analysis of the copolymer using ^{1}H NMR revealed successful copolymerization of the SCEMA monomer with PEGMEMA monomer, as was evident from the proton resonances around 4.55 and 2.83 ppm due to the carbonate methylene and succinimide protons, respectively (Figure 8.8). The presence of the protected maleimide group at the chain end was established by proton resonances at 6.54 ppm corresponding to the alkene protons of the oxanorbornene group and at 5.11 ppm belonging to the bridgehead protons. The incorporation ratio of the SCEMA and PEGMEMA monomers into the polymer backbone and molecular weight of the copolymer was estimated by the integration of the succinimide protons of SCEMA at 2.83, methoxy protons of PEGMEMA at 3.25 ppm and alkene protons of the maleimide-furan cycloadduct at 6.54 ppm. The experimentally obtained monomer compositions of the copolymer showed that the incorporation of the SCEMA monomer is lower than the composition in the feed. This examination was in agreement with the relatively lower reactivity ratio of SCEMA compared to the OEGMA.

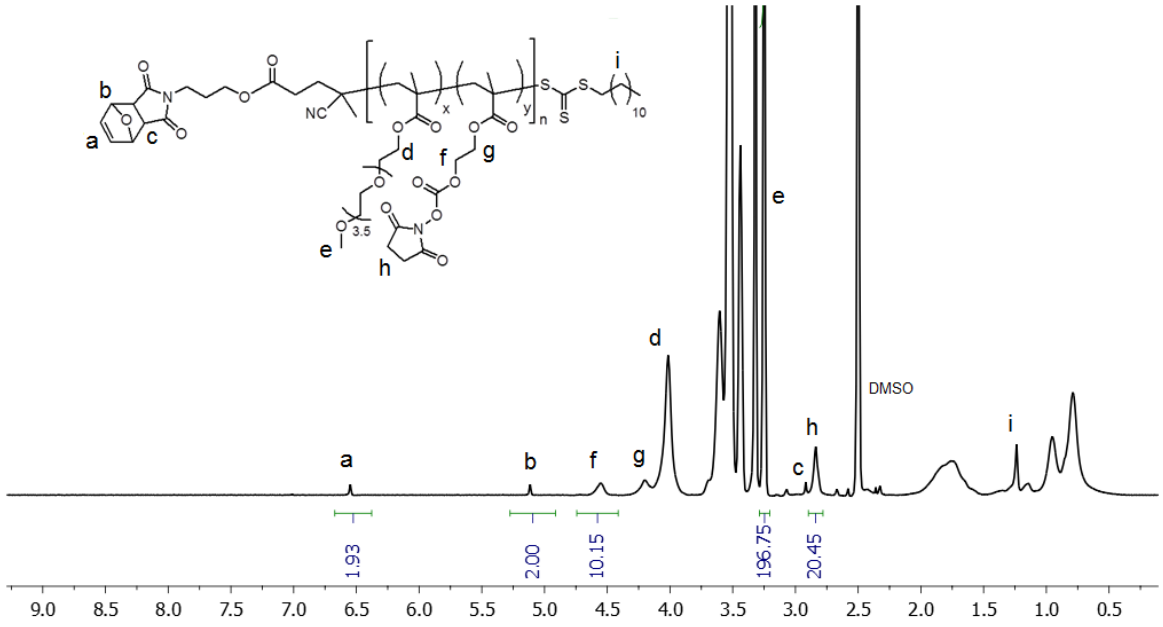


Figure 8.10. ^{1}H NMR spectrum of furan-protected maleimide end-functionalized copolymer FM-P.

The trithiocarbonate end group of the copolymer was removed via a radical cross-coupling reaction between the trithiocarbonate chain-end and AIBN. Radical cross-coupling reaction was performed at 70 °C in DMF for 4 h. A 25-fold excess of AIBN per RAFT group was used for the complete cross-coupling. After the purification of the polymer by precipitating into cold ether, the obtained polymer was subjected to retro Diels-Alder reaction to remove the furan protecting group and obtain the maleimide units in their thiol-reactive form (Figure 8.9). Polymer was dissolved in anhydrous toluene and refluxed at 110 ^{o}C to unmask the maleimide groups. Analysis of the obtained polymer M-P using ^{1}H NMR spectroscopy revealed that the removal of the furan protecting groups takes place in a quantitative fashion (Figure 8.10). Complete disappearance of the proton resonances of the bridgehead bicyclic structure and appearance of a new peak at 7.01 ppm due to the maleimide double bond was observed. There is a good agreement between the integration values of maleimide protons and pendant SCEMA and PEGMEMA monomer protons before and after the retro Diels-Alder reaction confirming the conservation of activated carbonate groups at side chains.

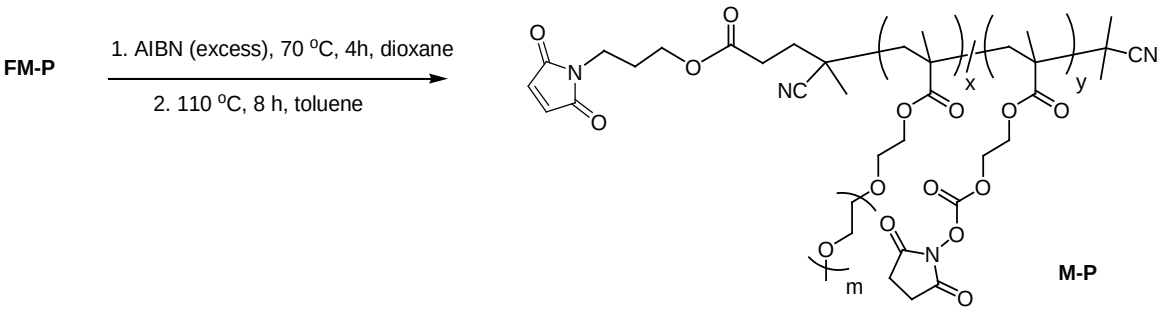


Figure 8.11. Synthesis of the copolymer M-P containing reactive carbonate side chains and maleimide end group.

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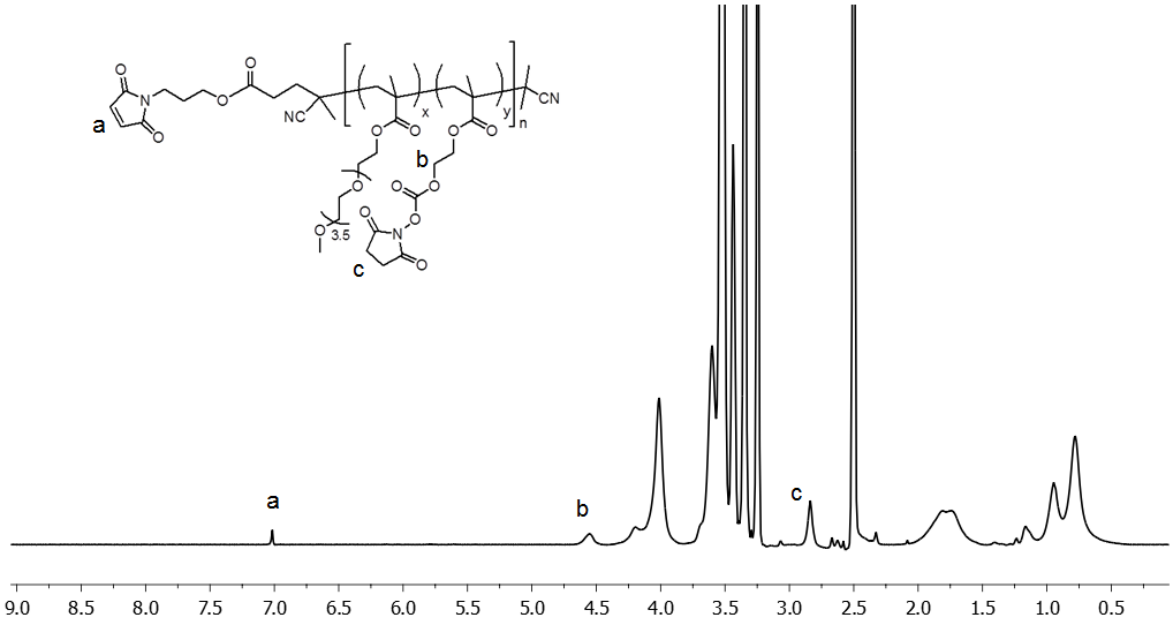

Figure 8.12. ^{1}H NMR spectrum of copolymer M-P.

8.3.3. Sequential Functionalization of Thiol and Amine Reactive Copolymer M-P

With the random copolymer M-P at hand, the sequential orthogonal functionalization was investigated via amine conjugation and nucleophilic thiol-ene addition. Due to the presence of NHS-activated carbonate groups, SCEMA-containing copolymer was expected to be functionalized by amines via carbamate formation. Since the scope of this project is based on the preparation of polymer-drug conjugates that carry drug molecules covalently attached with carbamate groups, an amine containing anticancer drug doxorubicin was attached to the side chain carbonate functionalities (Figure 8.11). The functionalization efficiency was evaluated by reacting copolymer M-P with doxorubicin in the presence of small amount of triethylamine in dichloromethane at 0 ^{o}C for 2 h. The relatively low

reaction temperature and reaction period was due to avoiding the maleimide group that is also capable of reacting with amines. Using 1.1 excess of amine per carbonate unit provided quantitative functionalization of the polymer as was evident from the disappearance of proton resonance at 4.55 and 2.83 ppm due to the carbonate methylene and succinimide protons, respectively (Figure 8.12). Newly formed proton signals belonging to the doxorubicin has also proved the successful conjugation. The maleimide signal retaining at 7.01 ppm shows that maleimide group stays intact during Dox attachment.

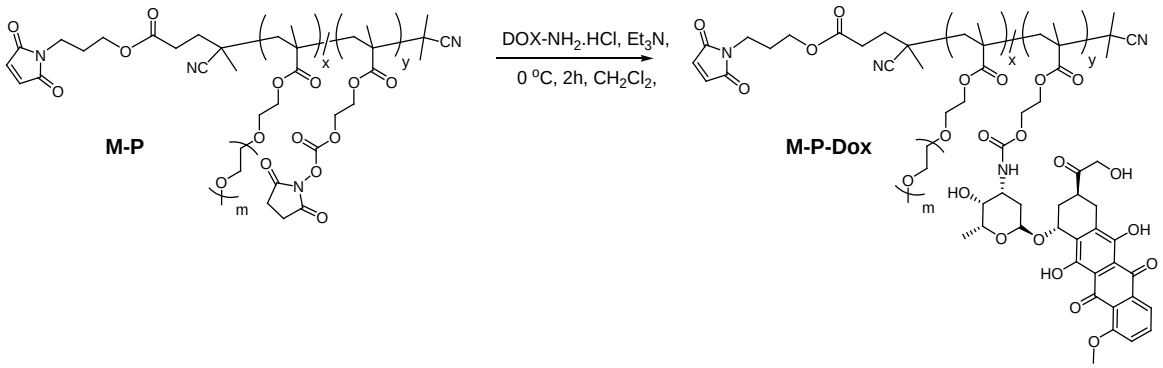

Figure 8.13. Functionalization of copolymer M-P with doxorubicin.

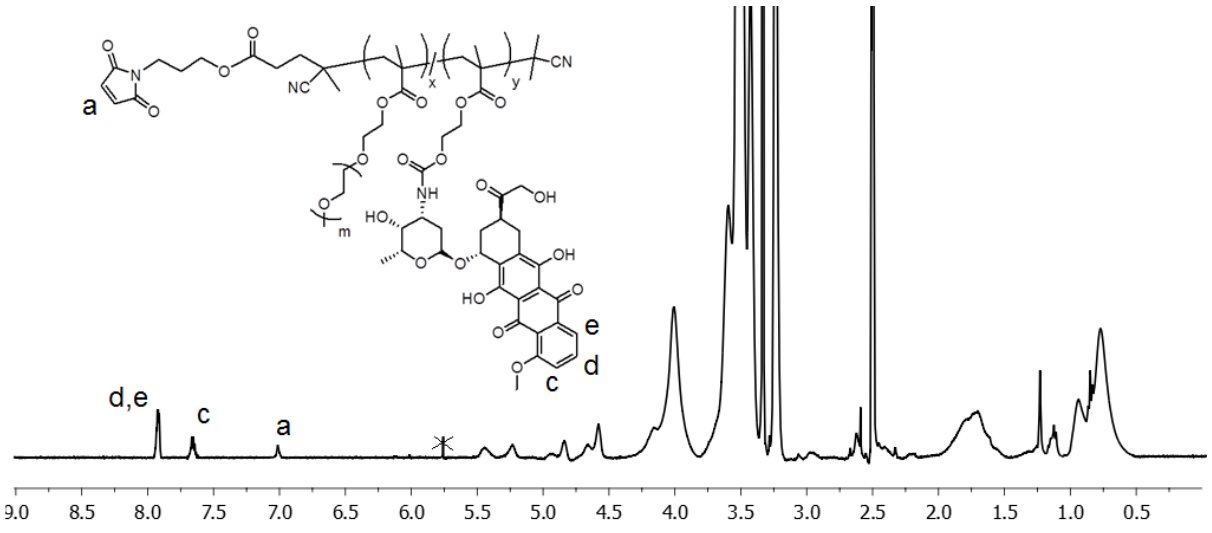

Figure 8.14. ^{1}H NMR spectrum of Dox-conjugated copolymer M-P-Dox.

The functionalization of copolymer M-P-Dox was investigated by conjugation of two different thiol containing molecules, folic acid thiol (FA-SH) and cyclic-RGD peptide (c(RGDfC)). Folic acid (folate or vitamin B9), is an important nutrient that it is required by living cells in the body for the biosynthesis of nucleotides and metabolic maintenance of

several carbon pathways [202]. FA also shows high binding affinity to the folate receptors (FRs), which are the proteins that captures and transports their ligands from the extracellular environment to inside the cell via endosomal pathways. Folate receptors are commonly overexpressed on many human cancer cell surfaces. Due to the high affinity of folate receptors to folate groups, folate-drug conjugates are widely utilized in drug delivery to deliver the conjugates into the cells via endocytosis based cellular uptake [203]. Beside the targeting group feature of FA, integrin-binding molecules such as Arg-Gly-Asp (RGD) peptides are also widely used for targeting $\alpha_v\beta_3/\alpha_v\beta_5$ integrins overexpressed in several angiogenic sites and tumors [204]. The doxorubicin conjugated polymer M-P-Dox with maleimide chain end functionality have thus evaluated in conjugation of FA-SH and c(RGDfC) targeting ligands.

The conjugation of folic acid thiol molecule to the maleimide end group was studied by reacting copolymer M-P-Dox with FA-SH. The nucleophilic thiol-ene addition reaction was carried out in DMSO at room temperature and catalytic amount of triethylamine was used to accelerate reaction (Figure 8.13). After the reaction, residual unbound FA-SH was removed by dialysis in DMSO and acetonitrile to obtain functionalized polymer F-P-Dox in pure form. According to ^{1}H NMR analysis, complete thiol conjugation was observed due to the disappearance of maleimide signals at 7.01 ppm and formation of new proton resonances belonging to folate molecule (Figure 8.14).

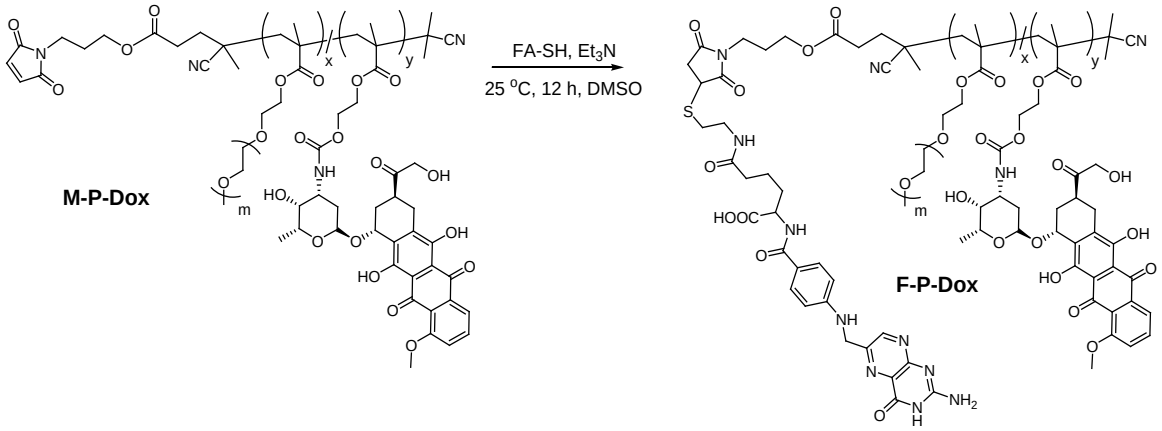

Figure 8.15. Conjugation of FA-SH onto copolymer M-P-Dox.

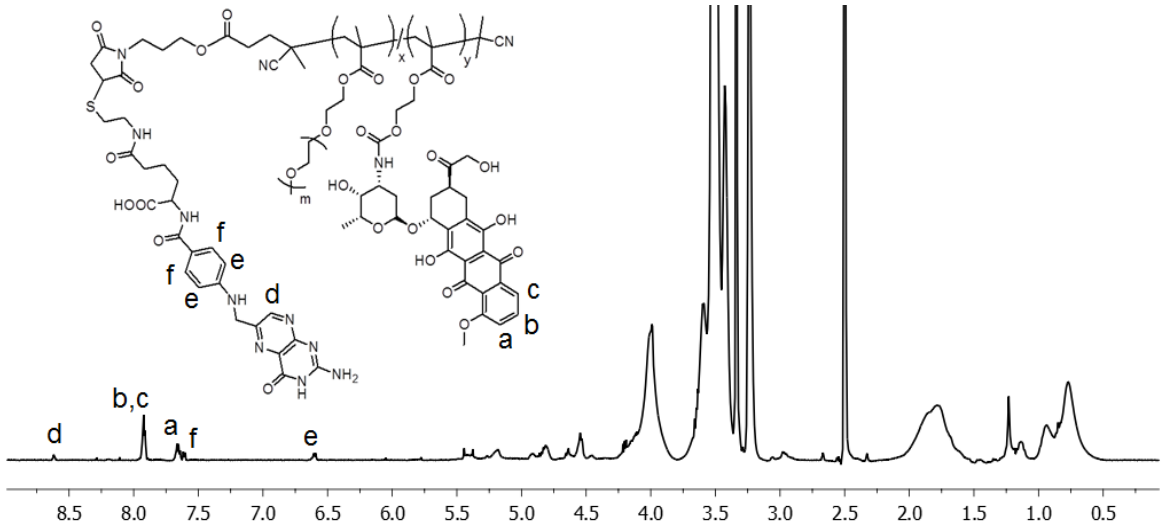

Figure 8.16. ^{1}H NMR spectrum of FA-SH conjugated copolymer F-P-Dox.

The copolymer M-P-Dox carrying maleimide end group was conjugated with thiol-functionalized cyclic peptide (c(RGDfC)) by employing thiol-maleimide nucleophilic addition reaction (Figure 8.15). The reaction was implemented out in DMSO at room temperature and catalytic amount of triethylamine was used to accelerate reaction. The purification of resulting polymer R-P-Dox was conducted by dialysis in DMSO and acetonitrile. In the ^{1}H NMR analysis, disappearance of the proton resonance at 7.01 ppm and the emergence of new peaks belonging to the cyclic peptide implied successful attachment of the c(RGDfC), via the Michael addition (Figure 8.16).

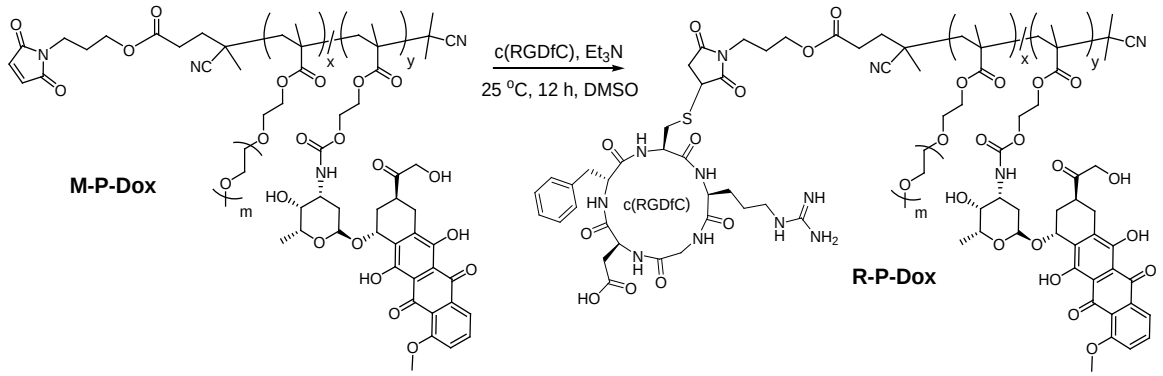

Figure 8.17. Conjugation of c(RGDfC) onto copolymer M-P-Dox.

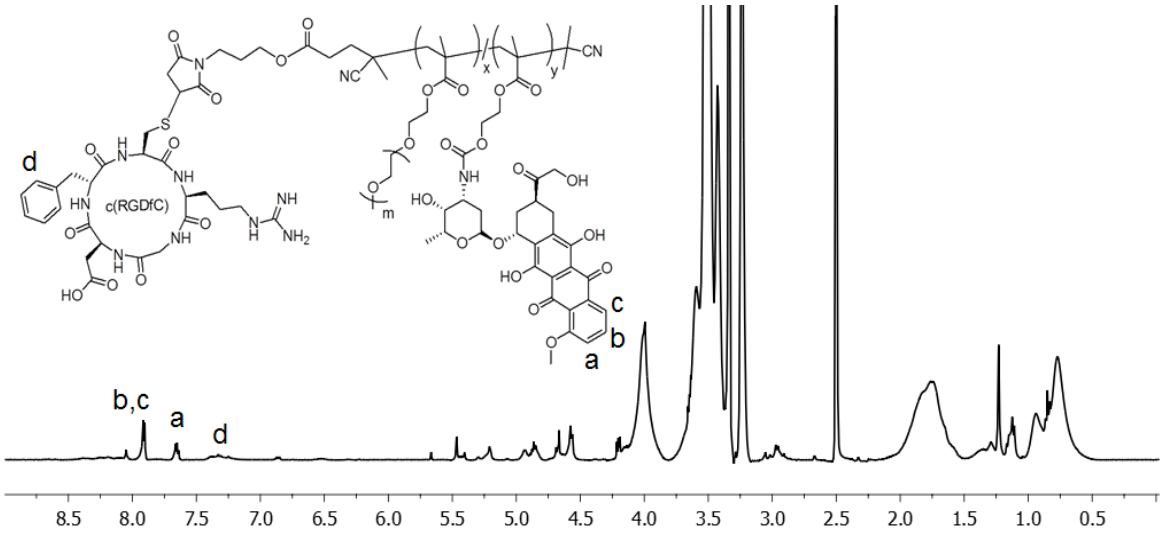

Figure 8.18. ^{1}H NMR spectrum of c(RGDfC) conjugated copolymer R-P-Dox.

8.3.4. Evaluation of Polymer-drug-targeting ligand Conjugates as Drug Delivery Platforms

In this study, the designed and synthesized copolymer with orthogonally functionalizable amine and thiol reactive groups was evaluated as a platform to prepare polymer based conjugates for drug delivery applications. By utilizing the activated carbonate side groups of polymer, we conjugated the anticancer drug doxorubicin via hydrolytically cleavable carbamate bond. Thus, we hypothesized that the conjugate would release its cargo via slow degradation of carbamate groups, providing a sustained release of the drug. Additionally, the attachment of two different targeting ligands has been considered as a mean of achieving target specific drug delivery. The obtained poly(PEGMEMA)-Dox-folic acid and poly(PEGMEMA)-Dox-c(RGDfC) conjugates were investigated in *in vitro* drug release.

Poly(PEGMEMA)-Dox-folic acid (F-P-Dox) and poly(PEGMEMA)-Dox-c(RGDfC) (R-P-Dox) conjugates that are subject to drug release via hydrolysis of carbamate bonds are incubated in acetate buffer (pH 5.0) and phosphate buffered saline (PBS, pH 7.4) solutions to study *in vitro* behavior. As shown in Figure 8.17, no initial burst release was observed for both FA and c(RGDfC) attached conjugates. This can be attributed to the chemical attachment of Dox molecules that their physical release from the polymer conjugates was eliminated. As expected, a pH-dependent release profile was observed for all conjugates. Higher drug release was observed with the lower pH value, depicting the

enhanced degradation of carbamate groups in more acidic medium. A slow and sustained drug release from conjugates was observed and over 30 days, the release of the drug was continued with slow kinetics.

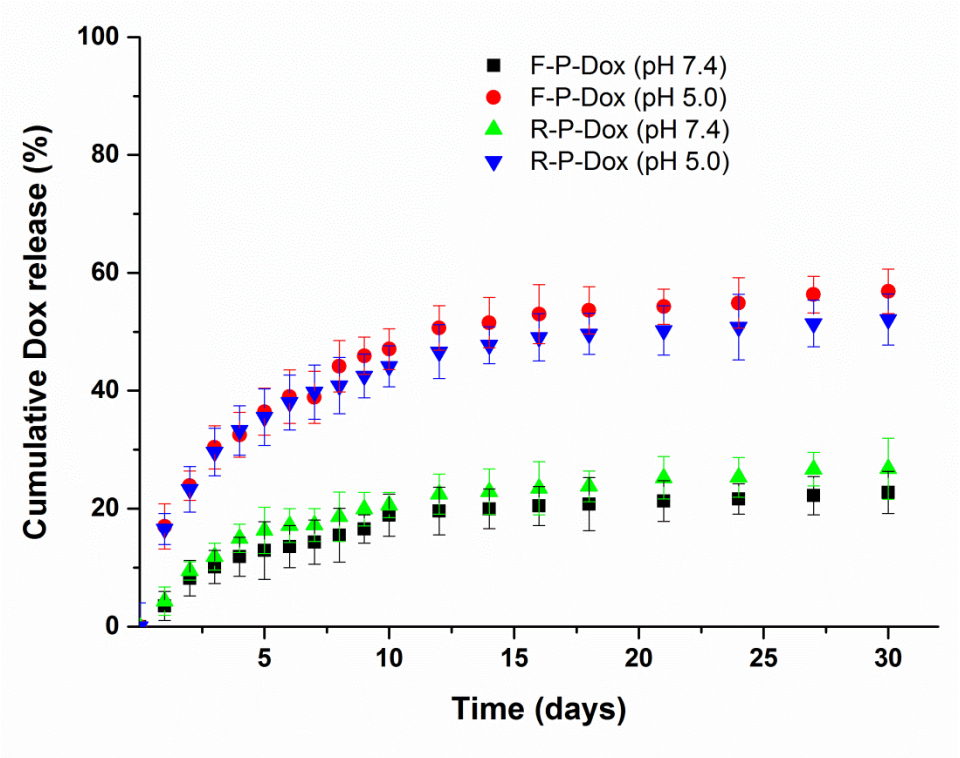

Figure 8.19. *In vitro* doxorubicin release from polymer-drug-targeting ligand conjugates.

8.4. Conclusion

Hydrophilic polymers incorporating NHS-activated carbonate group containing side-chains were synthesized using reversible addition fragmentation chain transfer (RAFT) polymerization. A novel activated-carbonate based monomer was utilized in synthesis of polymers with varying amounts of the functionalizable monomer and hydrophilic OEGMA-based monomers. A polymer bearing maleimide end group along with activated carbonate side chains was also synthesized and used in functionalization with thiol-bearing and amine-bearing bio-related compounds. Sequential functionalization of polymer was achieved by functionalization of side chain activated carbonate groups with an amine containing anticancer drug doxorubicin. Maleimide group at chain termini was also functionalized with folic acid and cyclic RGD peptide molecules through thiol-maleimide addition reaction. The functionalized copolymers with carbamate-bonded doxorubicin and

folic acid or cyclic RGD targeting groups were investigated as potential targeted drug delivery platforms. Polymer-drug-targeting ligand conjugates were evaluated in terms of *in vitro* drug release. According to *in vitro* drug release studies, a pH-dependent sustained release was observed for both folic acid and cyclic RGD containing conjugates. Cell internalization of conjugates on SKOV-3 (human ovarian cancer) and A549 (human lung adenocarcinoma) cell lines is currently being investigated.

9. CONCLUSIONS

This dissertation reports the design, fabrication and applications of novel hydrogels and soluble polymer-based macromolecular constructs that can find potential applications in various areas of biomedical sciences such as drug delivery. A variety of efficient ‘click’ type transformation based methodologies were utilized to synthesize novel polymers and hydrogels and efficient post-polymerization modifications were demonstrated to create functional materials.

In the first three projects of this dissertation, fabrication of CD-containing hydrogels with well-defined network structures and their use in target molecule conjugation and controlled drug release studies were reported. The last three projects of this dissertation explore synthesis of novel functional polymers that are amenable to facile and efficient post-polymerization modifications. Polymers bearing reactive units at their chain ends and/or as pendant side chains were synthesized using step growth and controlled radical polymerization techniques. Their post-polymerization modification to obtain functional soluble polymers and hydrogels were investigated.

Novel methodologies for synthesis of well-defined PEG-based hydrogels were explored using a thiol-functionalized cyclodextrin (CD) unit as a crosslinker. Hydrogels were synthesized using two different thiol-ene ‘click’ reactions, namely the radical thiol-ene and nucleophilic thiol-ene reaction. Hydrogels synthesized using photochemically induced radical thiol-ene crosslinking enabled access to micropatterned hydrogels. In comparison, the nucleophilic thiol-ene based methodology provided hydrogels without the need of any additional catalysts or thermal or photo-activation. The simplicity and rapid gelation kinetics suggest that the latter approach can be extended to obtain injectable hydrogels. In both approaches, clear and transparent hydrogels were formed with high gel conversions. Water uptake properties and viscoelastic gel behaviors of obtained hydrogels were shown to be dependent on the molecular weight of PEG segment and the crosslinker ratio. Hydrogels carrying functionalizable groups were obtained by varying the amount of CD-based crosslinker used in the gelation reactions. Hydrogels carrying residual alkene or thiol functional groups within the hydrogel matrix were efficiently functionalized with

thiol or maleimide-containing dyes and it was demonstrated that the extent of immobilization onto the hydrogels can be tuned by tailoring the amount of residual reactive groups in gel matrix. Additionally, drug absorption and controlled release studies from CD-containing hydrogels were tested using an anti-glaucoma drug, puerarin. The drug uptake and release properties were shown to be dependent on hydrogel composition.

Subsequent project outlines a methodology that extends the thiol-ene gelation system to enable fabrication of CD-containing hydrogels with tailored matrix. Novel poly(ethylene glycol)methacrylate based telechelic copolymers bearing thiol reactive alkene-based end groups were synthesized using RAFT polymerization. 2-hydroxyethyl methacrylate (HEMA) and/or di(ethylene glycol) methyl ether methacrylate (DEGMA)-based hydrophilic polymers were synthesized using reversible addition-fragmentation chain transfer (RAFT) polymerization. Thiol reactive maleimide and vinyl functional groups were placed at polymer chain end by using appropriate functional group containing CTAs and post-polymerization modification of existing polymers. Telechelic maleimide and alkene group containing polymers were also utilized in hydrogel fabrication. Obtained hydrogels comprising different RAFT polymers were characterized in terms of their physical properties such as water uptake capacity, surface morphology and rheological behaviors. Thereafter, these β-cyclodextrin containing hydrogels were evaluated for controlled release of puerarin. The methodology of synthesizing telechelic RAFT polymers depicted here may find potential application in polymer modification and design of hydrogel based controlled drug release systems.

To address the limited number of reactive groups in linear PEG-based system, side-chain reactive group containing linear PEG-based polymers were designed. Step growth polymerization was utilized to obtain linear PEG-based polytriazole copolymers containing thiol-reactive functional groups as pendant side chains. The copper(I)-catalyzed Huisgen-type 1,3-dipolar azide-alkyne cycloaddition reaction was utilized for the copolymerization of diazide and dialkyne functionalized monomers to install thiol reactive groups such as maleimide and strained bicyclic alkenes along the side chains. Polymers functionalization via click chemistry-based transformations and fabrication of chemically cross-linked functionalizable hydrogels was demonstrated.

Many applications demand coating solid planar and nanoparticle surfaces with polymeric layers. Fabrication of thiol-reactive polymeric coatings for nanoparticle modification was explored. Novel thiol-reactive polymers containing a catechol moiety was synthesized and application towards surface modification of magnetic nanoparticles using a ‘grafting-to’ method was demonstrated. The catechol functional group on the polymer chain-end allows fabrication of water dispersible magnetic nanoparticles that can be subsequently functionalized by thiol bearing molecules. One can envision that these thiol-reactive water soluble surface-anchorable polymers will find applications in many areas of biomedical sciences. As an example, modification of grafted polymers with a dye molecule furnished water dispersible fluorescent magnetic nanoparticles, a promising candidate for dual modality imaging applications.

In the final part of the thesis, an orthogonally functionalizable polymeric support that is reactive towards thiol as well as amine containing molecules is described. Polymers bearing maleimide end group along with activated carbonate side chains were synthesized and functionalization in an orthogonal fashion. In particular, this orthogonally reactive scaffold was utilized for preparation of polymer-drug conjugates. Sequential functionalization of polymers was achieved by functionalization of side chain activated carbonate groups with an amine containing anticancer drug doxorubicin. Maleimide group at chain terminus was functionalized with folic acid and cyclic RGD peptide molecules through thiol-maleimide addition reaction. Functionalized copolymers with carbamate-bonded doxorubicin and folic acid or cyclic RGD based targeting groups offer promising candidates as targeted drug delivery platforms.

In summary, a variety of efficient chemical transformations and coupling strategies were employed to fabricate and functionalize multifunctional polymeric materials. The facile fabrication and efficient functionalization of these novel macromolecular platforms makes them promising candidates for potential applications in several areas of biomedical sciences.

APPENDIX A: SPECTROSCOPY DATA

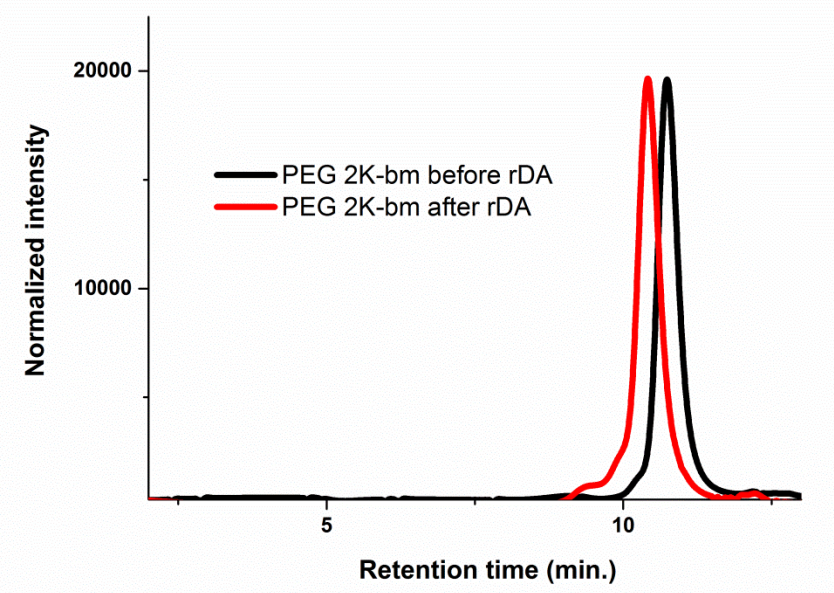

Figure A.1. SEC traces of PEG 2K-bm polymer before and after rDA reaction.

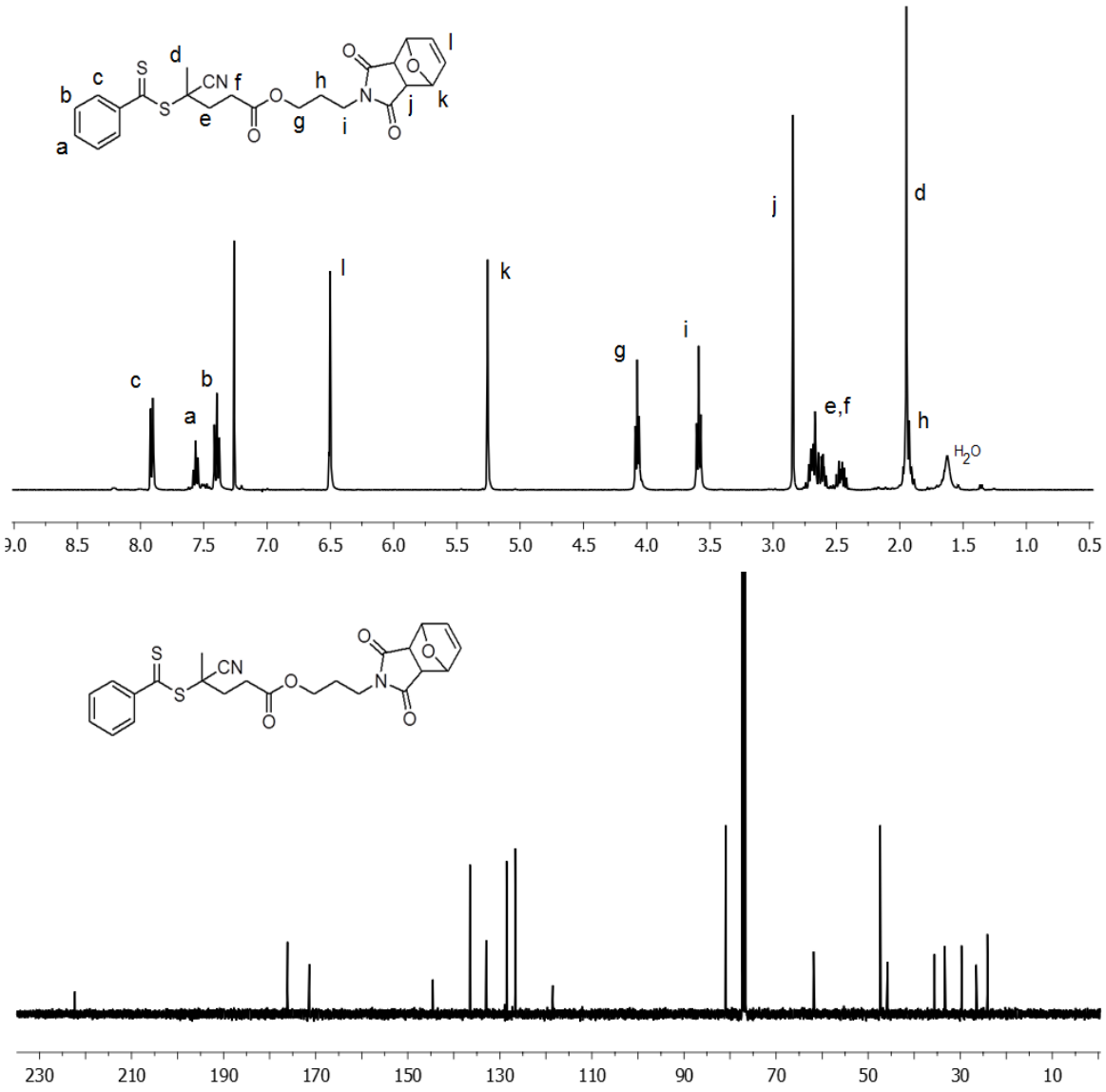

Figure A.2. ^{1}H and ^{13}C NMR spectra of CTA-FM.

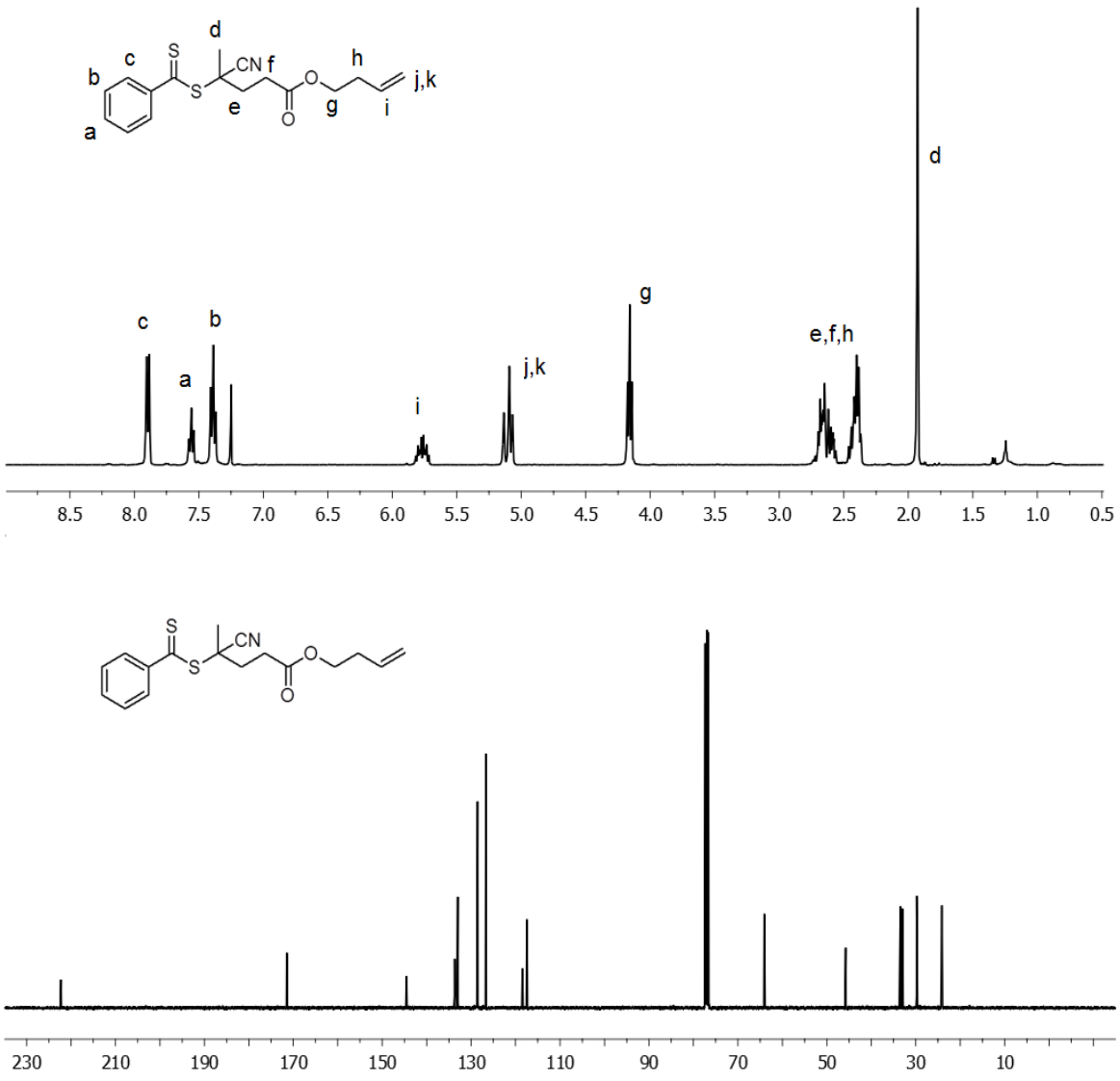

Figure A.3. ^{1}H and ^{13}C NMR spectra of CTA-V.

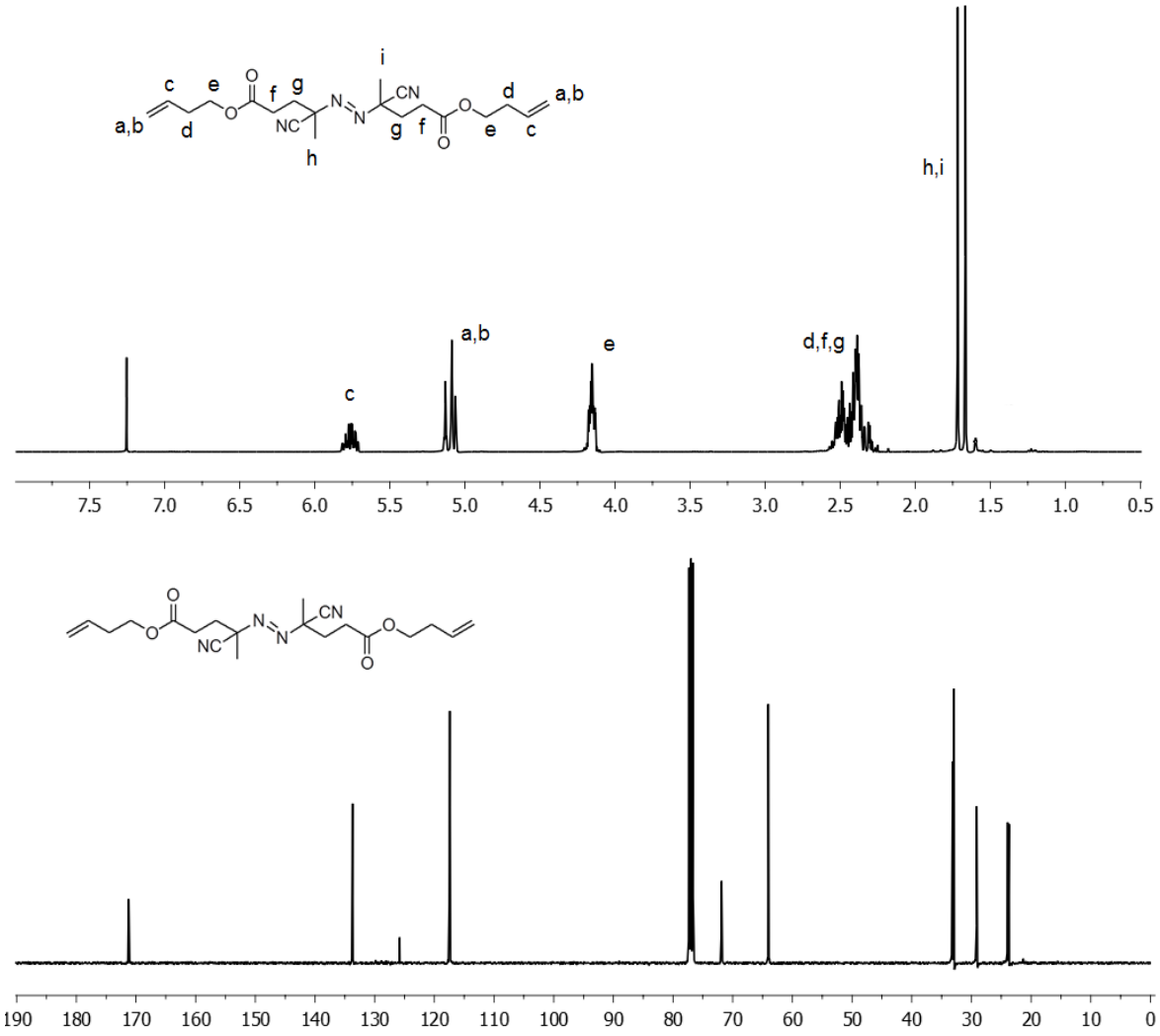

Figure A.4. ^{1}H and ^{13}C NMR spectra of Azo-V.

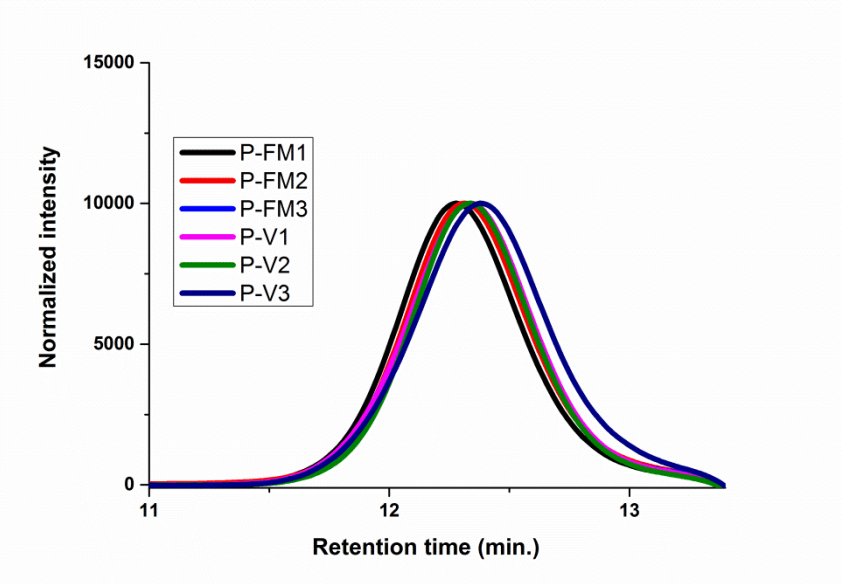

Figure A.5. SEC traces of RAFT polymers.

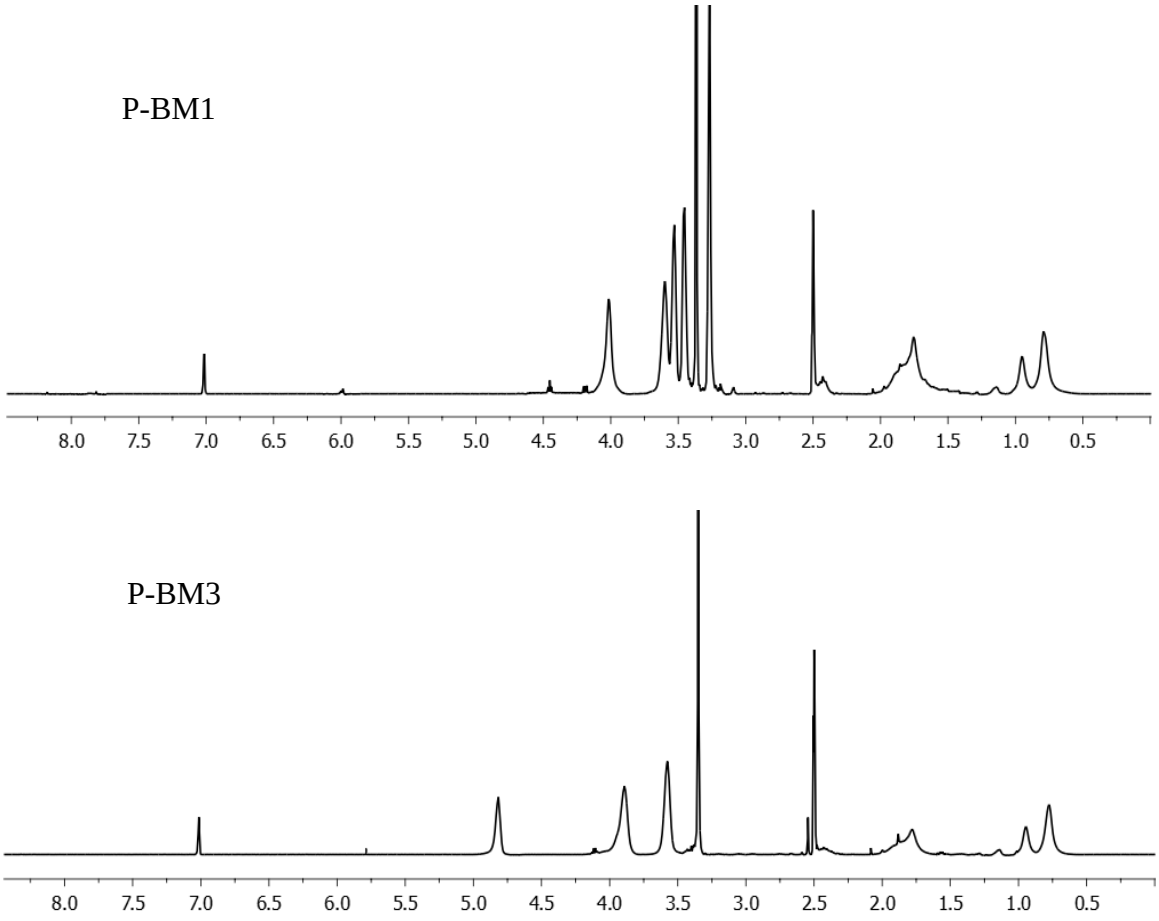

Figure A.6. ^{1}H NMR spectra of P-BM1 and P-BM3.

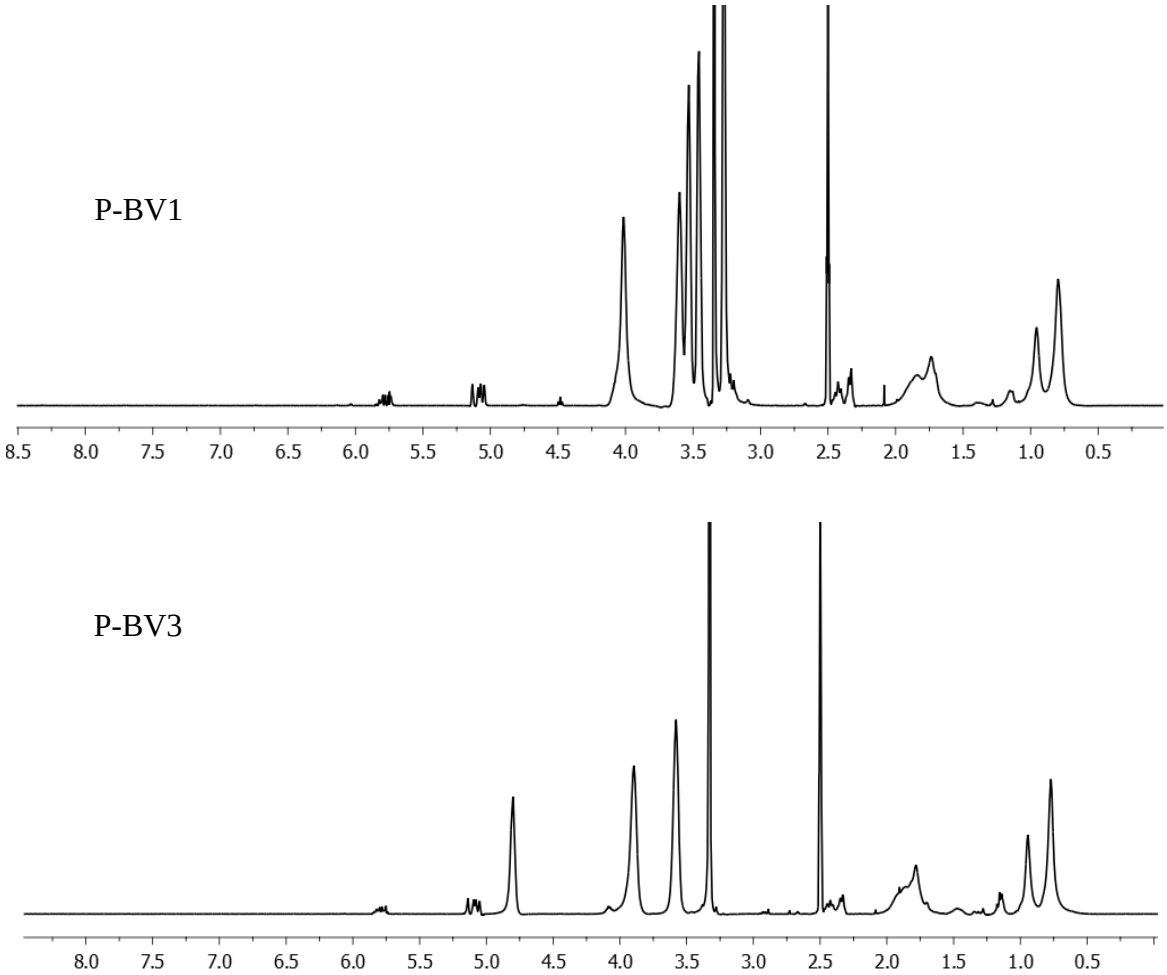

Figure A.7. ^{1}H NMR spectra of P-BV1 and P-BV3.

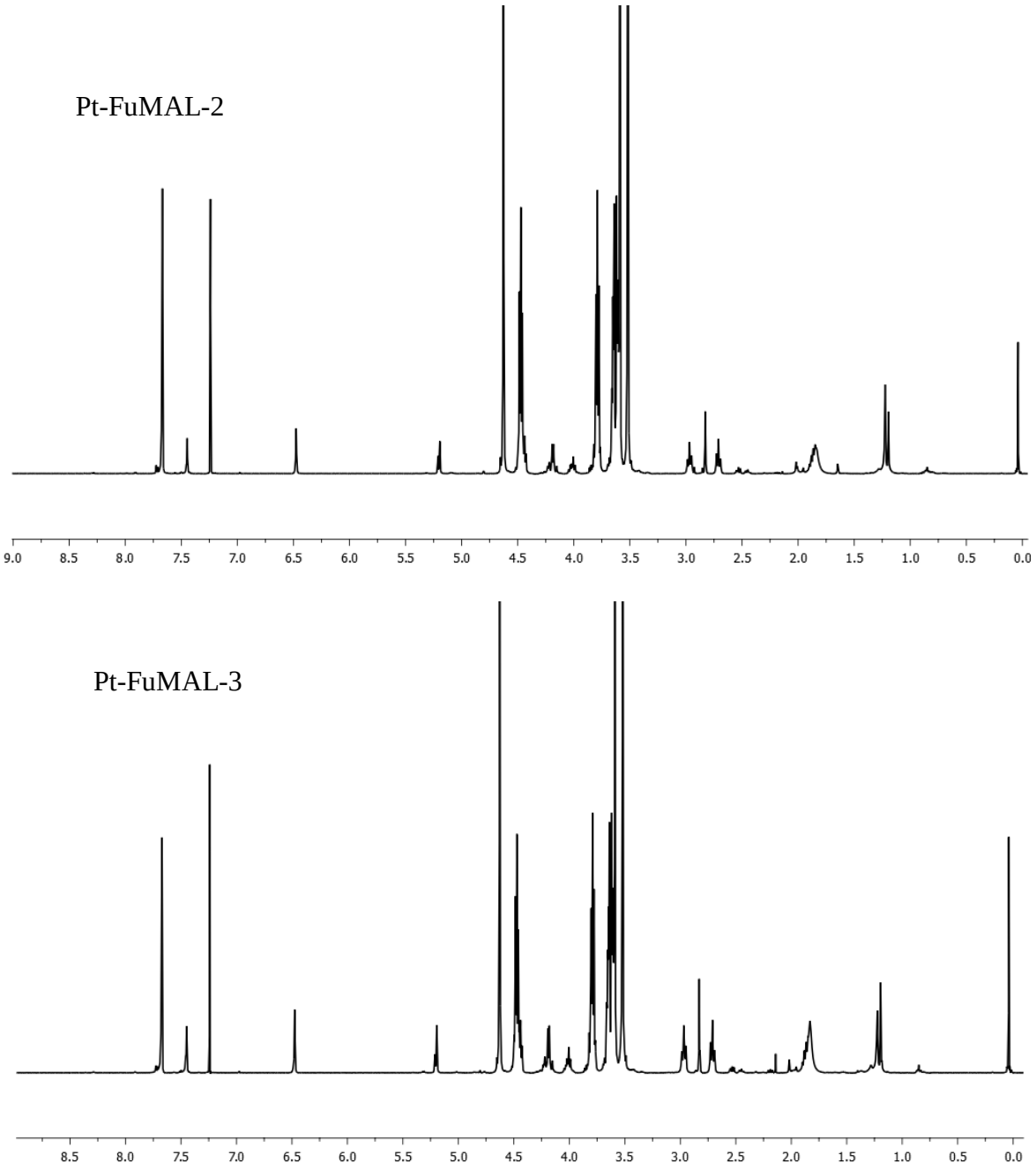

Figure A.8. ^{1}H NMR spectra of copolymers Pt-FuMAL-2 and Pt-FuMAL-3.

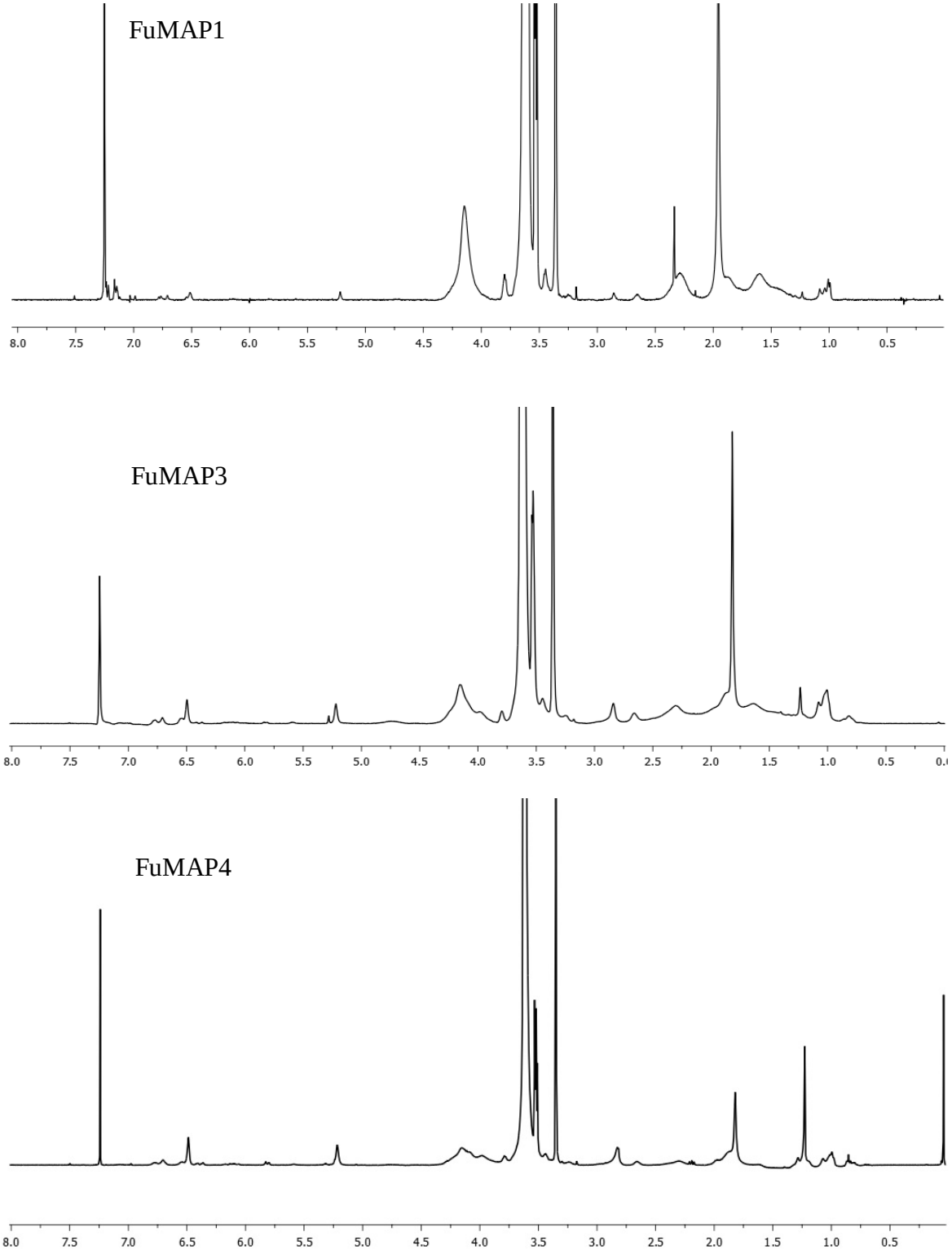

Figure A.9. ^{1}H NMR spectra of copolymers FuMAP1, FuMAP3 and FuMAP4.

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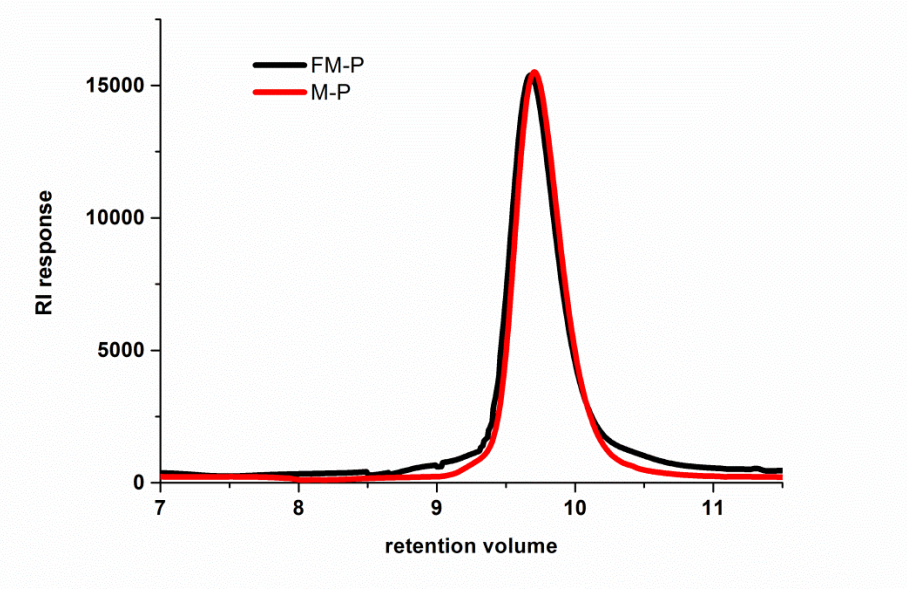

Figure A.10. SEC traces of copolymers FM-P and M-P.

APPENDIX B: PERMISSIONS FOR FIGURES

Title:	Fabrication of Maleimide Containing Thiol Reactive Hydrogels via Diels−Alder/Retro-Diels−Alder Strategy
Author:	Irem Kosif, Eun-Ju Park, Rana Sanyal, et al
Publication:	Macromolecules
Publisher:	American Chemical Society
Date:	May 1, 2010

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Order Content Author	Ke Peng,Itsuro Tomatsu,Alexander V. Korobko,Alexander Kros
Order Content Date	Oct 23, 2009
Volume number	6
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Expected completion date	Mar 2015
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Figure B.5. Permission for Figure 4.3.

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