THIOL REACTIVE POLYMERS AND HYDROGELS

by
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To My Beloved Grandmother

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LIST OF SYMBOLS / ABBREVIATIONS

AIBN	2,2'-azobisisobutyronitrile
ATRP	Atom transfer radical polymerization
BSA	Bovine serum albumin
$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Dichloromethane
D_2O	Deuterated water
DA	Diels-Alder
rDA	Retro Diels-Alder
DMF	Dimethylformamide
EtOAc	Ethyl Acetate
FT-IR	Fourier Transform Infrared
GPC	Gel Permeation Chromotography
HPLC	High Performance Liquid Chromatography
MeOH	Methanol
NMR	Nuclear Magnetic Resonance
PEG	Poly(ethylene glycol)
PEGDA	Poly(ethylene glycol) diacryalte
PEGMA	Poly(ethylene glycol) methacrylate
PEGMEMA	Poly(ethylene glycol) monomethyl ether methacrylate
TEA	Triethylamine
TEG	Triethyleneglycol
TGA	Thermogravimetric Analysis
THF	Tetrahydrofuran

ABSTRACT

THIOL REACTIVE POLYMERS AND HYDROGELS

Synthesis of polymers and hydrogels with reactive side chains has attracted considerable attention since these polymers are widely utilized in bioconjugation. We have recently introduced the synthesis of polymers decorated with maleimide units as 'reactive' functional groups utilizing of a novel methacrylate monomer which accommodates a masked maleimide functionality. This thesis expands on the polimerizations of this abovementioned monomer. Since the synthesis of such reactive polymers with narrow molecular weight distributions is desirable for many applications, Atom Transfer Radical Polymerization (ATRP) has been employed to obtain such polymers. Poly (ethylene glycol) (PEG) based polymers are known to be non-immunogenic and biocompatible and are promising candidates for formulation of polymer-drug conjugates. As one part of this study, water soluble PEG methacrylate based copolymers that contain thiol reactive maleimide side chains have been synthesized using ATRP. The maleimide groups are directly incorporated during the polymerization using a furan protected maleimide containing monomer. After the polymerization, the maleimide groups can be activated to their reactive form to furnish the thiol reactive copolymers. The maleimide side chain containing polymer can be efficiently derivatized by any thiol containing molecule under mild, reagent free aqueous conditions.

Hydrogels are used in a wide variety of areas such as biomolecular immobilization, tissue engineering, sensors, implant coatings, and drug delivery. Such increased interest also necessitates synthesis of novel hydrogel materials to widen the scope of intended applications of such materials. With this motivation, PEG methacrylate based hydrogels containing thiol reactive maleimide functional groups directly incorporated into the hydrogel have been synthesized. During the polymerization, the thermal deprotection of the maleimide groups in some of the monomers resulted in the formation of an *in situ* crosslinker. After gelation, the remaining protected maleimide groups could be activated to their reactive forms via a thermal

activation step. The successful incorporation of maleimide groups and covalent functionalization of the hydrogel were investigated using fluorescent labeling with thiol containing dye molecules and enzymes.

ÖZET

TİOL BAĞLANABİLEN POLİMER VE HİDROJELLER

Yan dallarında reaktif grup bulunduran polimerlerin ve hidrojellerin sentezi, biyomoleküllerin çapraz bağlanması için uygun olduklarından çok ilgi çekicidir. Yakın geçmişte reaktivitesi gizlenmiş bir maleimid monomeri kullanarak, reaktif grup olarak maleimid fonksiyonel grubunu barındıran polimerler sentezlemiştik. Bu tezde bahsedilen monomerin polimerleşme tepkimelerini genişletmiştir. Bu tip reaktif polimerlerlerin moleküler ağırlık dağılımları dar olacak şekilde sentezlenmesi arzu edildiği için Atom Transfer Serbest Radikal Polimerleşme (ATRP) yöntemi kullanılmıştır. Polietilen glikol (PEG) bazlı polimerler immun sistemini uyarmayan ve biyouyumlu olarak bilinirler, ve polimere bağlı ilaç formülasyonları için umut vaad eden adaylardır. Bu çalışmanın bir kısmı, tiol bağlanabilen maleimid yan zincirleri içeren, PEG metakrilat bazlı, suda çözünebilen kopolimerlerin ATRP ile sentezlenmesini içermektedir. Maleimid grupları, furan korumalı maleimid içeren monomerlerin kullanılmasıyla polimerleşme tepkimesine doğrudan dahil olurlar. Polimerleşme tamamlandıktan sonra, maleimid grupları tiol reaktif kopolimerleri elde etmek üzere aktif hale getirilebilirler. Maleimid yan dallı polimerler, ağır tepkime koşulları ve kimyasal eklenmesi gerekmeden, hatta sulu ortamlarda verimli bir şekilde tiol içeren moleküllere dönüştürülebilirler.

Hidrojellerin biyomoleküllerin sabitlenmesi, doku mühendisliği, sensörler, implantasyon ve ilaç salınımı gibi geniş kullanım alanları vardır. Bu alana artan ilgi, bu tip malzemelerin kullanım alanlarını genişletmek için yeni hidrojel sentezlerini gerektirmiştir. Bu gereklilikle, tiol bağlanabilen maleimid fonksiyonel grupları içeren PEG metakrilat bazlı hidrojeller sentezlenmiştir. Maleimid grupları, furan korumalı maleimid içeren metakrilat monomerlerin kullanılmasıyla hidrojele kopolimerizasyon sırasında doğrudan dahil olurlar. Bazı monomerlerdeki maleimid gruplarının koruma gruplarının polimerleşme sırasında çıkması çapraz bağlayıcının oluşmasına sebep olmuştur. Jelleşmeden sonra, kalan korunmuş

maleimid grupları termal olarak aktif hale getirilebilirler. Maleimid gruplarının hidrojel yapısına katılması ve hidrojellerin kovalent olarak fonksiyonelleştirilmesi tiol içeren boya molekülleri ve enzimler kullanılarak florasan etiketleme ile incelenmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1. Reactive Polymers as Soluble and Solid Support

The use of proteins and peptides as human therapeutics has attracted attention in recent years since novel peptides and proteins have been discovered and their mechanism of action in vivo is better understood. However many of the pharmaceuticals based on use of proteins and peptides suffer from short circulating half-life, immunogenicity, proteolytic degradation, and low solubility. In order to overcome these pharmacokinetic and pharmacodynamic limitations many strategies are being applied such as manipulation of amino acid sequence to decrease immunogenicity and proteolytic cleavage, conjugating to natural or synthetic polymers [1, 2].

Conjugation of proteins to poly (ethylene glycol) (PEG) is widely investigated and some of the examples of PEG-protein drugs were introduced to the market in early 1990s [3]. The coupling reaction between PEG and the proteins generally requires mild chemical conditions. The most common reactive groups in polypeptides are nucleophiles such as thiol, alpha amino group, carboxylate and hydroxylate. The carboxylic groups can not be easily activated without having reaction with the protein amino groups, which results in intra or inter molecular crosslinking. Early works in literature involve the activation of hydroxyl terminal group of PEG to react with amino groups on the lysine residues [4].

Haddleton and coworkers have reported polymers designed to react with amine (from lysine and N-terminal α-amino residues) functionalities present at the surface of proteins. They investigated the use of new N- Succinimidyl ester and α-aldehyde-functional poly (methoxy PEG) methacrylates obtained via Cu(I) mediated living radical polymerization and application of these polymers in conjugation reactions with a model protein, lysozyme. Multi site attachment was observed in both studies [5, 6].

In light of many such endeavours, it was realized that it is beneficial to perform site-specific conjugation through cysteine residues which are not participating in disulfide bond. The free cysteine residues are rarely present in proteins; however, they can be generated via reduction of disulfide bonds and by genetic engineering methods [7]. Although thiol reactive polymers with terminal maleimides, vinyl sulfones, iodoacetamides, and activated disulfides have been synthesized to achieve conjugation to proteins, there are very few of polymers with thiol reactive groups on the side chain. Among these available thiol reactive functional groups, maleimide appear to be an attractive candidate since they are very stable, yet reactive under appropriate conditions.

1.1.1. Bioconjugation with Reactive Polymers

Haddleton and co-workers disclosed two retrosynthetic approaches to obtain maleimide end functionalized macromolecules to target cysteine residues on the proteins [8]. They introduced the maleimide moiety into the polymers by following two independent approaches: (a) a post functionalization of a preformed primary amine-terminated polymer (Figure 1.1), and (b) the use of a "protected" maleimide initiator for the polymerization step followed by deprotection to give the expected maleimide terminated polymers (Figure 1.2). These α-functional methacrylate polymers have been succesfully employed in coupling reactions with a tripeptide and a model protein Bovine Serum Albumin (BSA).

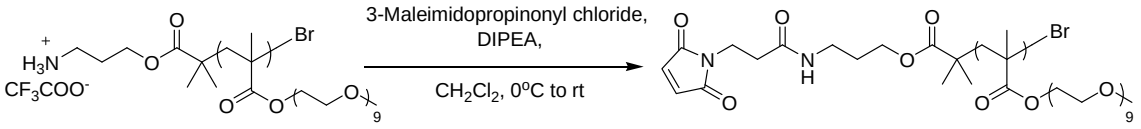


Figure 1.1. Post functionalizaiton of 1^{o} amine terminated polymer

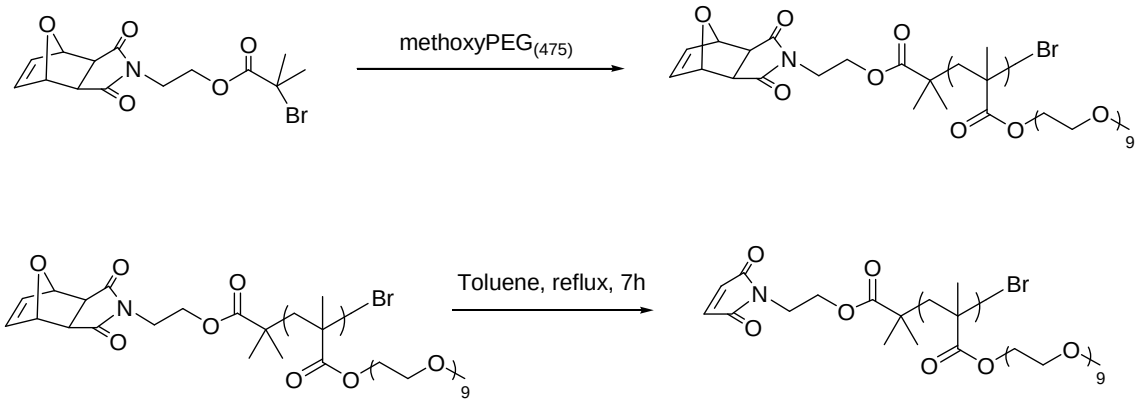


Figure 1.2. Synthesis of maleimide terminated polymer

In a recent study, Maynard et al. utilized the ATRP and atom transfer coupling reaction (ATR) to prepare polystyrenes with maleimide groups at both ends to enable reaction with thiol containing molecules such as cysteine [9]. An ATRP initiator with protected maleimide functionality was prepared and used to polymerize styrene. Semitelechelic styrene polymers were dimerized through ATR coupling. Resulting telechelic polymer was conjugated to *N*-acetyl-L-cysteine methyl ester after heating to activate maleimide groups (Figure 1.3).

Most bioconjugates are macromolecules comprised of one peptide and at least one polymer chain, depending on the number of the (poly) peptide reactive sites. However, in some cases, it is beneficial to have multiple peptides in a polymer chain. In order to achieve this, in 2001 Müller, Brocchini and coworkers reported the synthesis of well-defined poly(N-methacryloyloxysuccinimide)s (PNMS) utilizing ATRP and conjugation was investigated with GG-β-naphthylamide hydrobromide as a linker- drug model compound [10]. The remaining, active ester groups were reacted with 1-amino-2-propanol in order to obtain N-(2-hydroxypropyl) methacrylamide (HPMA) units (Figure 1.4). However, it has been reported

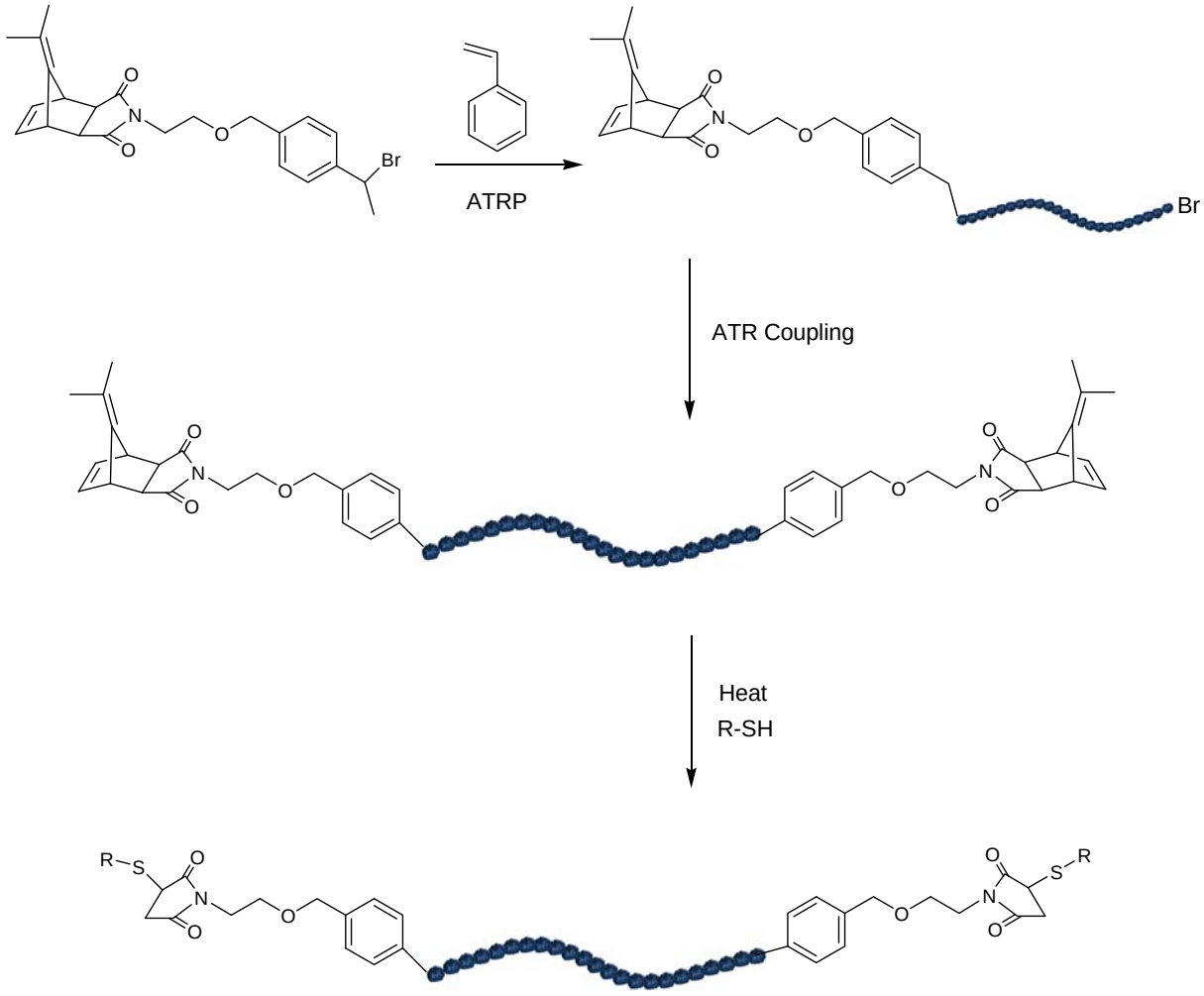


Figure 1.3. Synthesis of telechelic maleimide polymer by ATR coupling

that the post-polymerization modification of poly(N-methacryloxysuccinimide) resulted in side reactions, which include ring opening of the succinimide moiety and ringclosing attack of amides on neighboring active esters resulting in glutarimide formation [11].

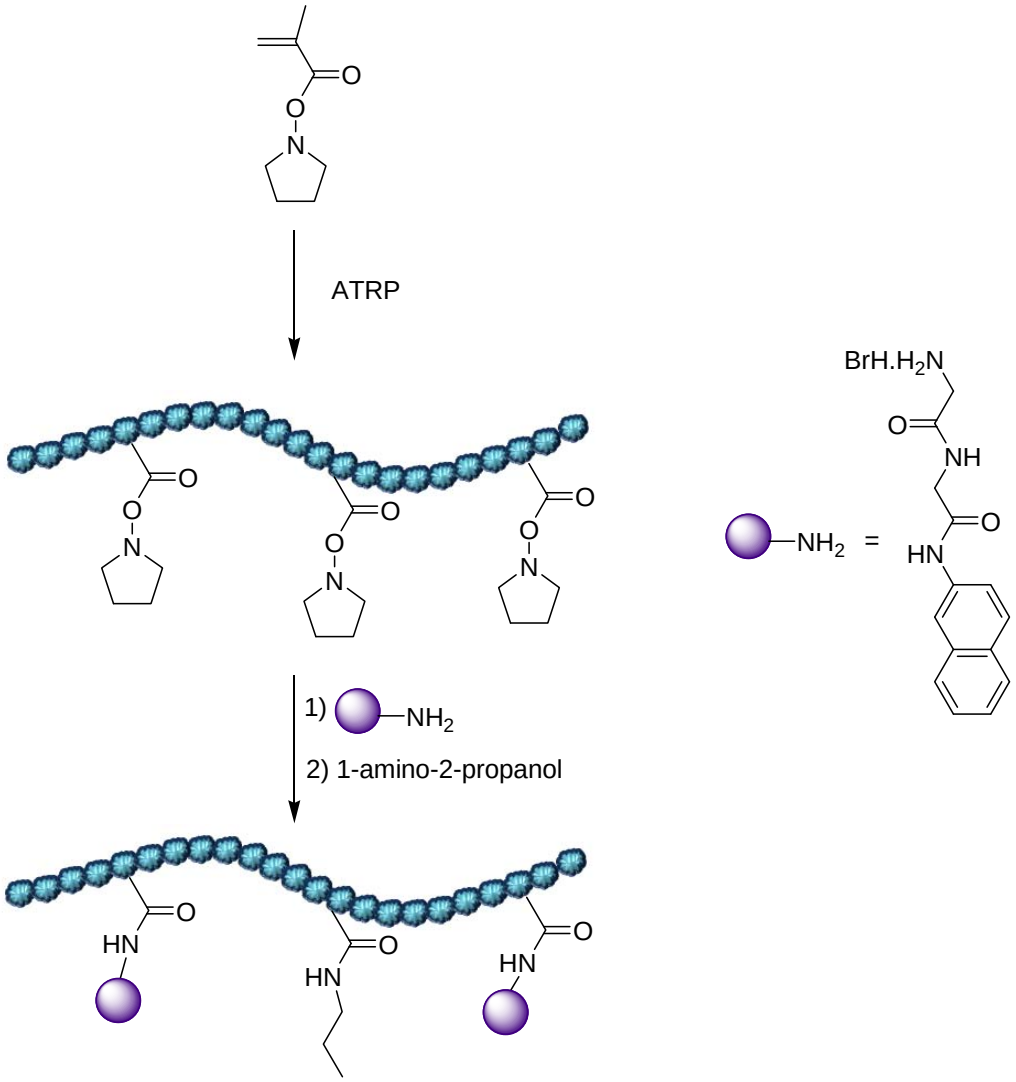


Figure 1.4. Synthesis of side chain amine reactive polymers

1.1.2. Bioconjugation on Solid Support

Chemoselective immobilization of biomolecules onto solid surfaces is another attractive area where polymers with reactive groups are widely utilized. Immobilization of biomolecules

onto solid surfaces is at the root for many biological applications such as microarrays, microbeads, nanoparticles, biosensor chips and surface modification of medical devices.

Numerous strategies have been reported to modify surfaces with polymers including self-assembled monolayers (SAMs) and polymer brushes which are 'grafted to' or 'grafted from' the surface. Klok and coworkers reported an interesting route to covalently attach a protein to a polymer-grafted surface using surface-initiated ATRP in a several-step approach [12]. 3-(2-bromoisobutyramido) propyl(trimethoxy)silane was grafted onto the surface as an ATRP initiator and three different methacrylate monomers (HEMA, PEGMA360 and PEGMA526) were polymerized. The hydroxyl groups of the brush repeat units were activated with p-nitrophenyl chloroformate (NPC) in order attach O6-benzylguanine (BG) leading to BG-functionalized brushes. The BG-functionalized brushes are used to chemoselectively immobilize *O*6-alkylguanine-DNA-alkyltransferase (AGT) fusion proteins with a defined orientation and surface density (Figure 1.5). In this example, the reactive groups were incorporated as a post-polymerization modification of the polymer brushes.

In 2006, an study that highlights the potential use of maleimide group in biomolecular immobilkization was repotrted by Howorka and co-workers. The researchers prepared glass surfaces carrying a dense layer of maleimide tethered poly(ethylene glycol) (PEG) which are potential platforms for DNA oligonucleotide micro arrays [11]. The glass slides were first silanized by using 3-glycidoxypropyl trimethoxysilane (GPS). The epoxide groups of the GPS-silanized surface were hydrolyzed to diols which were subjected to oxidative cleavage resulted in the formation of aldehyde slides (Figure 1.6). To obtain a homogeneous thin layer of PEG, a solution of PEG-diamine was applied onto the slides. PEG-grafted slides displaying terminal amino groups were incubated with succinimidyl 4-[p-maleimidophenyl] butyrate resulted in maleimide terminated surfaces which were then used to immobilize DNA oligonucleotides. Although the study demonstrated succesful application of such maleimide

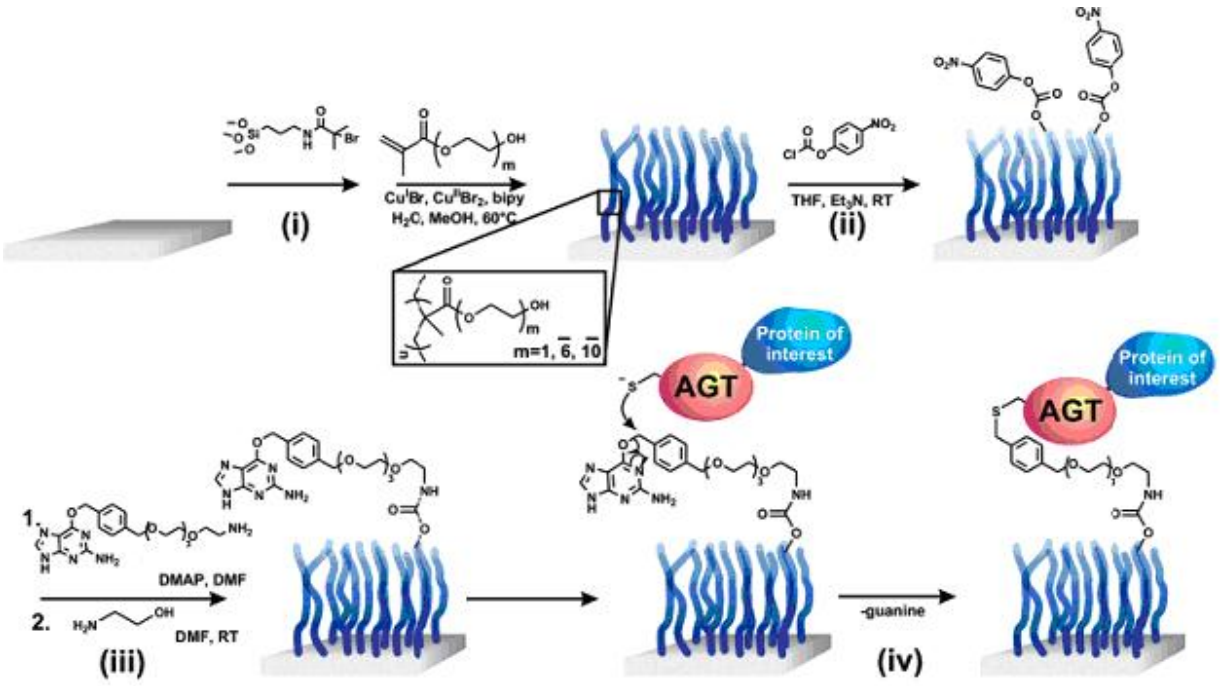


Figure 1.5. Synthesis of polymer brushes for immobilization [12]

terminated polymers attached onto solid surfaces for DNA immobilization, the synthesis of such material was multistep. A direct access to such reactive polymers would be certainly advantageous.

Reactive polymers also found application in the area of generation of patterns of biologically active molecules such as peptides, DNA, biotin on solid substrates. These materials possess potential applications in the design of microarrays and biochips. Challenges in creation of microfabricated arrays involve addressing controlled and selective immobilization of molecules in defined positions on a surface. In the development of rapid, robust screening micro arrays, several strategies have been succesfully applied such as robot-based high-precision contact printing [13], multistep photolithography [14], selective molecular assembly patterning [15], and soft lithographic approaches, such as the micro contact printing (μCP) method.

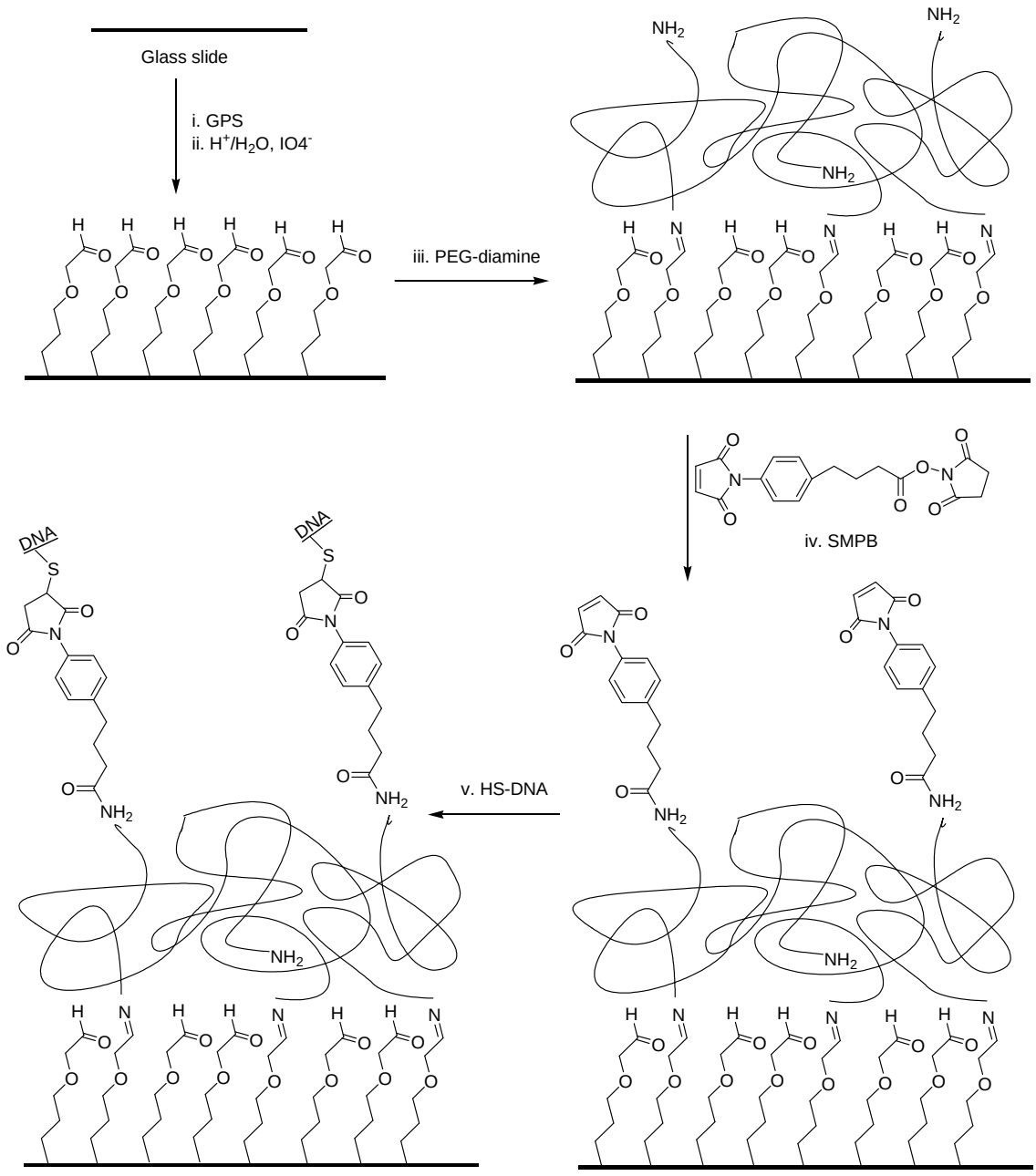


Figure 1.6. Schematic representation of the procedure for preparing PEG-grafted, maleimide terminated surfaces for the immobilization of thiolated DNA oligonucleotides

The μCP technique which was developed by Kumar and Whitesides [16], is widely used for the fabrication of monolayer-based micrometer- and sub-micrometer patterns due to the simplicity of the method. Low cost, flexibility, and the possibility to pattern curved substrates are among other advantages that make μCP very attractive for a variety of applications such as the development of certain biosensors [17]. A study related to patterning of biomolecules onto reactive polymeric surface was recently reported by Yon and coworkers. They reported the synthesis of polymer SAMs on Si/SiO_2 wafers utilizing random copolymers obtained by radical polymerization of poly(ethylene glycol) methyl ether methacrylate (PEGMA), 3-(trimethoxysilyl)propyl methacrylate (TMSMA), and *N*-acryloxysuccinimide (NAS). PEGMA was chosen as a comonomer due to its known antibiofouling property. The ability of the pSAMs to block nonspecific adsorption of proteins was evaluated against bovine serum albumin as a model protein. They observed that immobilization of the protein could be optimized by arranging the ratio of bio-reactive *N*-acryloxysuccinimide monomer and PEGMA [18].

1.2. Reactive Hydrogels

Hydrogels are polymeric networks that are capable of absorbing water. The high water content renders hydrogels biocompatible and due to their resemblance to natural tissues, hydrogels have become materials of interest in a wide variety of fields such as biomolecular immobilization, tissue engineering, sensors, implant materials and implant coatings, and drug delivery [19]. Such increased interest necessitates synthesis of novel hydrogel materials to widen the scope of intended applications of such materials.

The use of hydrogels as scaffolds for various applications derives from their ability to encapsulate various guest molecules in their interior. These guest molecules can be growth factors or signaling peptide molecules for various types of cell culture and tissue engineering applications or drug molecules that can be slowly released into the vicinity of an implant. The functionalization of the hydrogels can be accomplished either by physiabsorption or by covalent attachment of the molecule of interest. Physiabsorption is limited by the lack of

control over rate and uniformity; in addition, it is not applicable to incorporation of cell adhesion peptides. For this reason, hydrogels bearing immobilized biomolecules have a great potential of use in tissue engineering.

To covalently immobilize biomolecules on hydrogels usually a monomer of the desired biomolecule is synthesized. For example West et al. synthesized a photochemically crosslinked hydrogel scaffold with covalently immobilized gradients of basic fibroblast growth factor (bFGF) using PEG diacrylate and acryloyl-PEG-RGDS. It was observed that cells seeded on this hydrogel were aligned in the direction of increasing bFGF concentration within 24 hours [20].

In a later study, West and coworkers synthesized hydrogels containing covalently attached biotin and the cell adhesive peptide RGDS. They utilized a PEG monomer which contains an activated ester at one terminus that allows attachment of any molecule of interest using the amidation chemistry, while the acrylate group at the other end allows covalent integration into the hydrogel and created 3D patterns using single photon absorbance (SPA) photolithography [21].

An alternative and more convenient strategy is to immobilize biomolecules on hydrogels via post–functionalization since it leads to circumvent the need of converting molecules of interest such as peptides or oligonucleotides into reactive acrylates. There are not many examples on this approach since the synthesis of reactive hydrogels is limited due to lack of functional group tolerance with the conventional free radical polymerization methods.

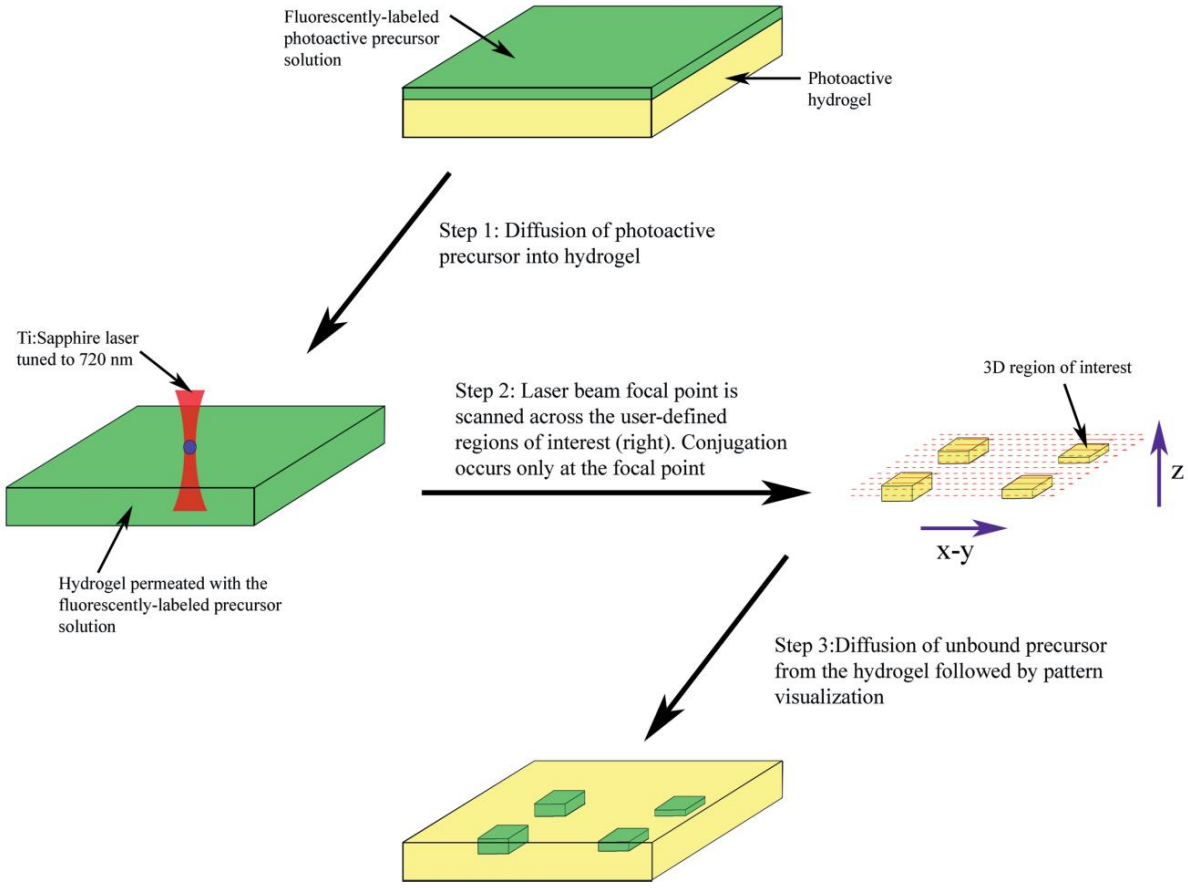


Figure 1.7. Photoinduced patterning of poly(ethylene glycol) diacrylate network

In a pioneering study, Shoichet et al. have shown the synthesis of hydrogel matrix containing photolabile protected sulphydryl groups. Upon spatially controlled photodeprotection of photosensitive S-2-nitrobenzyl-cysteine (S-NBC), the reactive thiol groups were utilized to immobilize maleimide labeled biomolecules such as fluorescein-tagged-maleimide terminated GRGDS peptide unit (Figure 1.8a). Immobilization of biomolecules in selected volumes in a 3D hydrogel matrix was achieved by using laser fabrication techniques and photochemistry (Figure1.8b). Adhesion channels provided oriented axonal growth in hydrogels, suggesting the potential use of biochemical channels into biodegradable hydrogel matrices in clinical applications for guided nerve regeneration or be extended to other tissues [22].

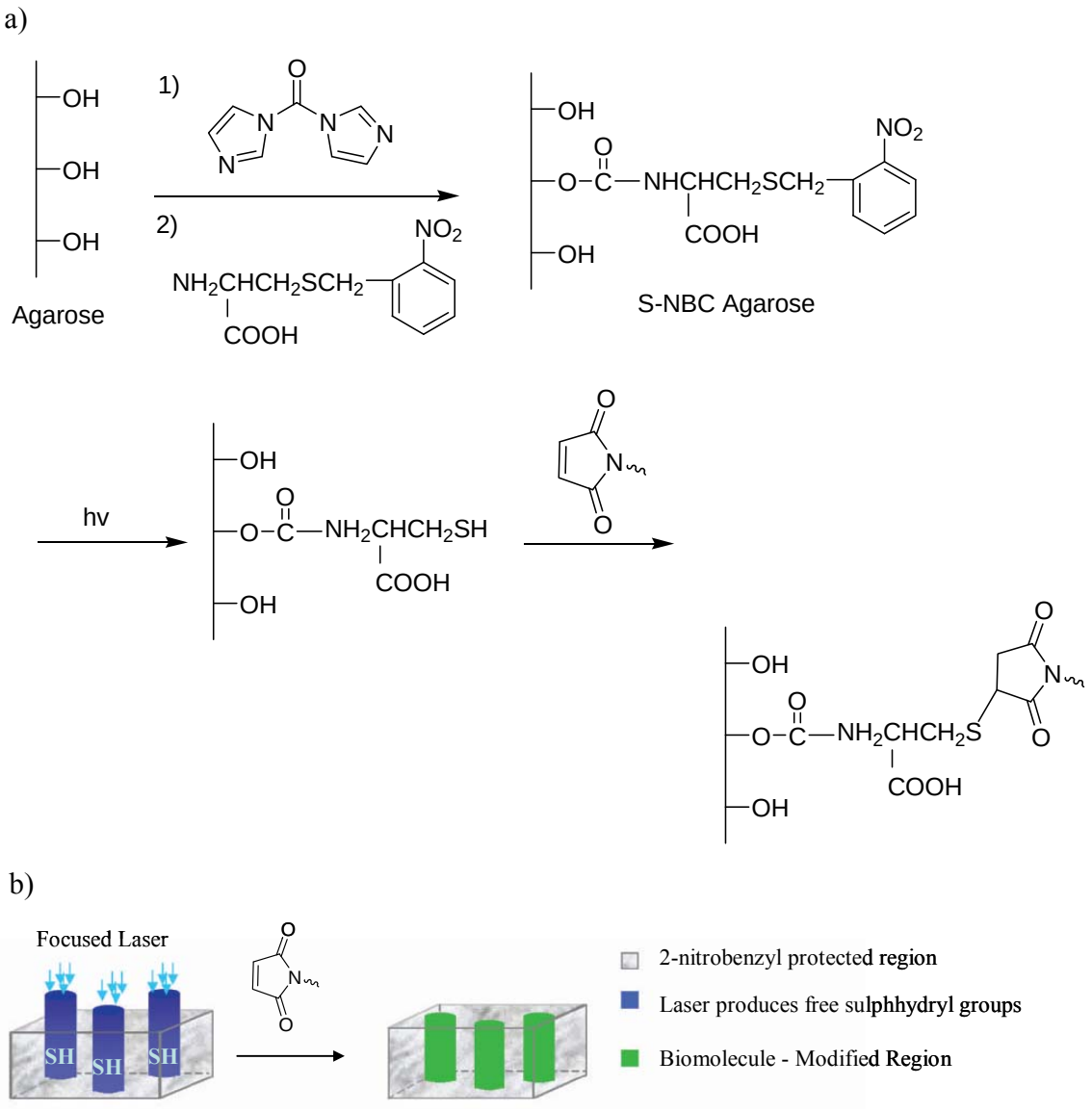


Figure 1.8. a) Synthesis of maleimide reactive hydrogels, b) Creation of biomolecular channels in hydrogel matrix

In 2006, Anseth et al. demonstrated the bioconjugation of apoptosis-inducing anti-Fas IgG Mabs to reactive PEGDA hydrogels synthesized via the photopolymerization of PEGDA and N-hydroxysuccinimide-PEG-acrylate. Antibodies react with NHS through the amine

groups present in their structure. This system has been demonsrtated to reduce the immune response to transplanted tissues compared to systems with obtained with passive encapsulation technology [23].

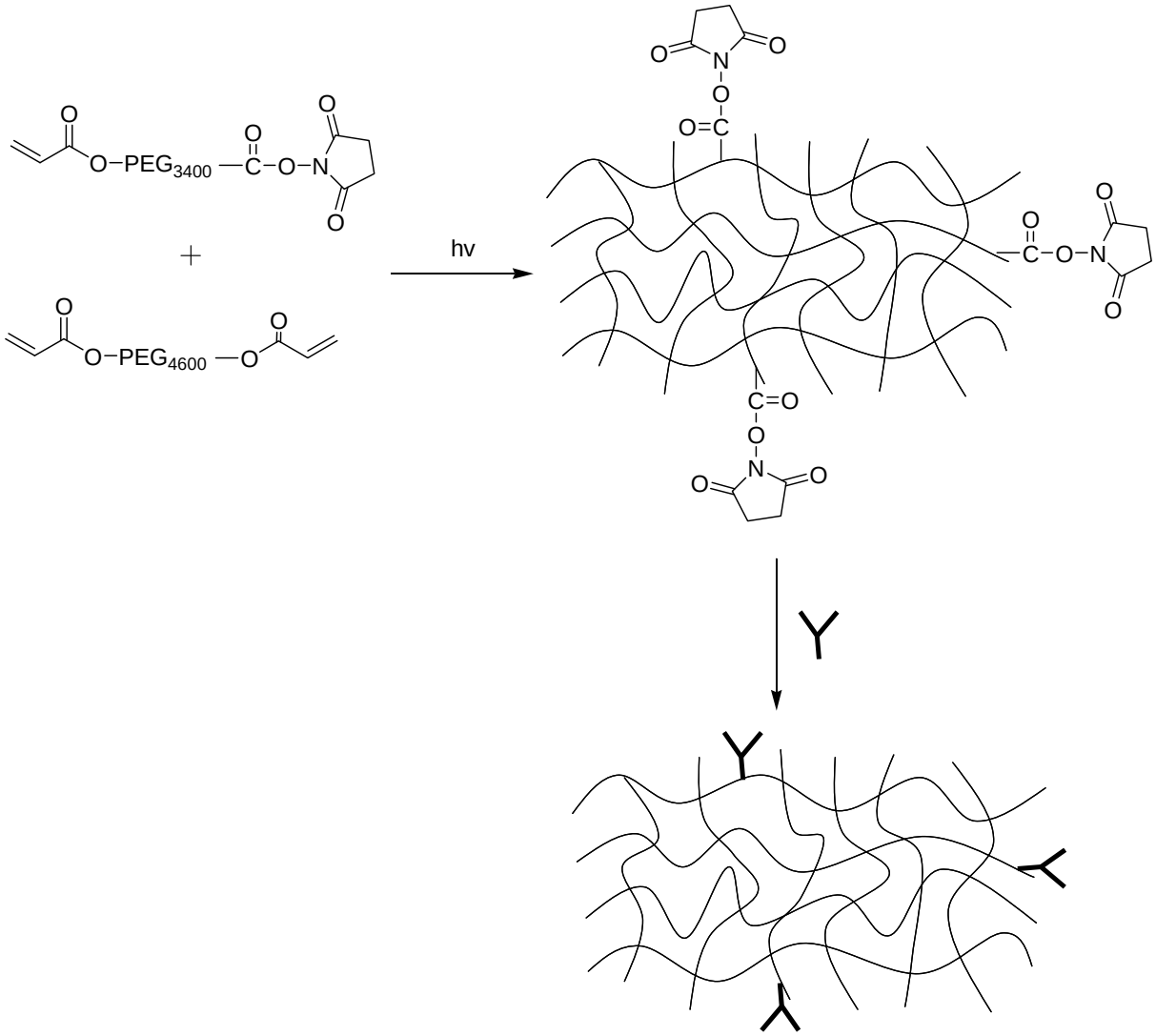


Figure 1.9. Synthesis of amine reactive hydrogels

An alternative approach for synthesizing hydrogels for biomolecule immobilization is the addition of reactive comonomers such as glycidyl methacrylate which renders the obtained hydrogel reactive towards functionalization using electrophilic oxirane ring opening reactions.

Suter et al. synthesized poly(ethylene glycol)-dimethacrylate based hydrogels by introducing a small weight fraction of glycidyl methacrylate (GMA) through photopolymerization [24].

Survey of literature revealed that thiol reactive hydrogels have not been synthesized, eventhough a much sought after functionalization method for immobilization of biomolecules to polymers or hydrogels is via thiols as cysteine residues can be engineered at specific sites. Cysteines undergo facile reactions with maleimides, orthopyridyl disulfide units, vinyl sulfones and iodoacetamides. Although the maleimide group has been extensively exploited in biomolecular immobilization using mono layers on various metallic and glass surfaces, examples of polymeric materials with maleimide side chains have been very limited. The limitation stems from the tendency of the reactive double bond in maleimide to participate in radical polymerization. Recently, remarkable advances have been made in this area by utilization of protected maleimide based initiators and monomers to obtain polymers with maleimide as their end groups. As discussed later, we have recently introduced a Diels-Alder reaction based maleimide protection-deprotection strategy in order to obtain multiple attachments of biomolecules to polymer side chains [25].

1.3. Diels-Alder Reaction in Polymer Chemistry

The Diels-Alder (DA) reaction is one of the most widely used synthetic methods in which an electron-rich “diene” functional group reacts with an electron-poor “dienophile” unit via [4+2] cycloaddition reaction [26,27]. In this cycloaddition reaction, addition of a dienophile to a conjugated diene results in a cyclic product referred as an adduct (Figure 1.10). The Diels- Alder reaction can be carried out under mild conditions in high yields without the formation of byproducts. The high efficiency and modular nature of Diels-Alder reaction has included this reaction to the click chemistry concept which was introduced by Sharpless and co-workers in 2001 [28]. Furan and anthracene derivatives have been widely explored as diene components in macromolecular construction. Due to their high dienophile character and wide structural variability through the nature of the nitrogen substituents, maleimides are preferred as the choice of dienophile (Figure 1.10). One of the most relevant and attractive aspects of the

DA reaction is the thermoreversibility, which implies that the adducts can be readily converted to the starting material via a cyclo reversion reaction known as retro-Diels-Alder (rDA) reaction or retrodiene reaction [29].

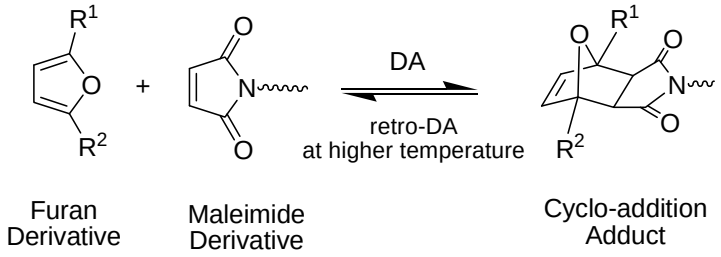


Figure 1.10. Representation of the DA and rDA reactions

The effectiveness and uniqueness of Diels–Alder chemistry have attracted attention of many research groups and the reaction has been widely used in the design of macromolecular structures such as dendrimers and polymers. In polymer chemistry, the DA reaction has been utilized to synthesize linear polymers as well as crosslinked structures. Linear polymers have been synthesized via successive DA cycloadditions involving multifunctional diene and dienophiles as the monomer, e.g. a di-furan derivative and a bismaleimide. For the crosslinked macromolecules, DA reaction have been utilized to induce the cross-linking to the polymer structure by taking the advantage of the inter-macro-molecular couplings with a difunctional complementary reagent such as furan copolymer plus bismaleimides or polymer bearing maleimide moieties plus difurans. The thermal sensitivity of the DA reaction makes it an attractive candidate for thermally reversible cross-linking. These polymers can revert to their precursors through the rDA reaction and this feature can be exploited in many applications, like the possibility of recycling or “mending” network-based materials.

1.3.1. Diels-Alder Reaction in Polymerization

DA “click” chemistry is a suitable method for the synthesis of polymers; it has been widely used for the synthesis of a diversity of polymers in the past. The DA reaction in

polymer chemistry dates back to an early report about formation of oligomers of cyclopentadiene by Alder and coworkers in 1932. In 1961, Stille and co-workers accomplished the use of DA reactions in polymerizations to produce high molecular weight polymers [30]. In this study, they utilized the DA reaction between cyclopentadiene based dimers and bismaleimides. The study did not promise further developments due to the utilization of biscyclopentadienyl compounds that are highly prone to homopolymerize.

In 1990s, a variety of polymers such as polyimides, polyurethanes and acrylic copolymers have been synthesized utilizing DA reaction [31-32]. A series of papers which show the DA polymerization between difuran derivative and bismaleimide have been published in the early nineties. Gandini's group with their broader and long-standing interest in furan polymers has been engaged in different polymer applications of the Diels-Alder reaction involving furanic structures. In one of their more recent studies, they investigated the DA polymerization of a difuran diacetal with either aliphatic or aromatic bismaleimides [33] (Figure 1.11). This AA & BB type monomers were polymerized via Diels-Alder type polycondensation in THF yielding the polymer.

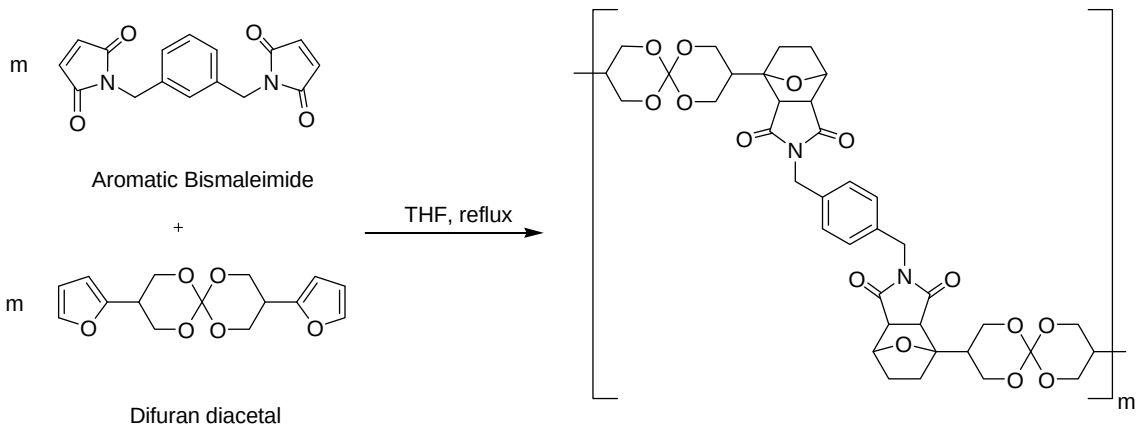


Figure 1.11. Diels-Alder polycondensation of difunctional monomers

Wüdl et. al.synthesized dendritic polymers using DA cycloaddition in 2002 [34]. They investigated the properties of thermally re-mendable crosslinked polymeric material obtained by DA reaction between a furan-based compound and a maleimide derivative (Figure 1.12). This dendritic polymer was prepared in 3 h at 75 °C with 95 % yield. Thermal treatment at 150 °C for 15 minutes resulted in approximately 30 % of disconnection (irreversible below 120 °C) of the adduct, while heating at 80 °C for an hour provides reconnection of the linkages.

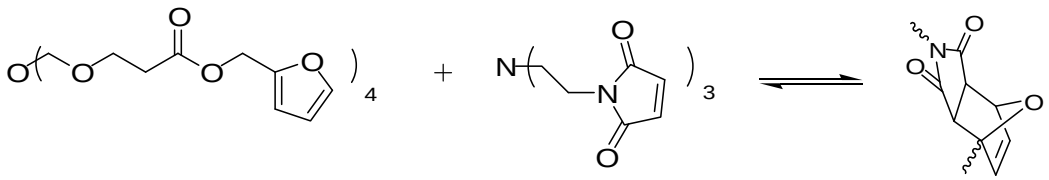


Figure 1.12. Synthesis of dendritic polymers

1.3.1.1 Use of Diels-Alder Reaction in Synthesis of Reactive Polymers: A subclass of polymers is dendrimers. Dendron polymer conjugates are rapidly becoming common macromolecular building blocks in both biopharmaceutical and materials research. A recent study from our research group introduced a modular approach towards the synthesis of polymers dendron conjugates (Figure 1.13). In this study Diels-Alder cycloaddition between the anthracene containing polymer and latent reactive dendrons containing masked maleimide groups at the core leads to quantitative functionalization of the polymer chains to afford dendronized polymers [35].

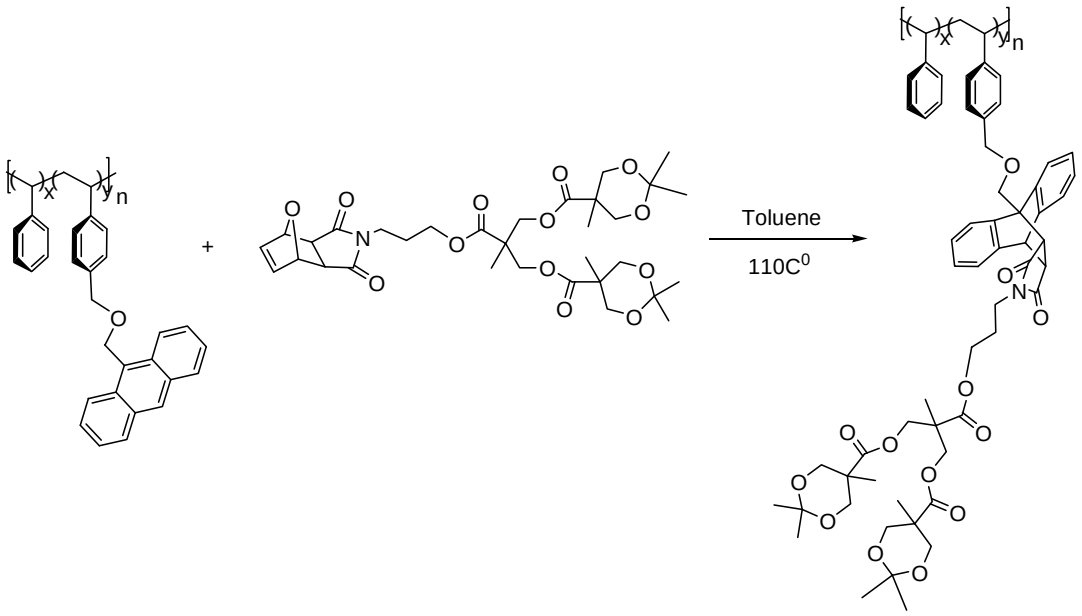


Figure 1.13. Synthesis of polymer-dendron conjugate

Polymers that can be functionalized via Michael-type addition reaction can be synthesized by utilizing monomers with masked maleimide functionalities. In 2006 Bailey and Swager used furan masked maleimides to synthesize rhodamine modified poly(phenyleneethylenes) (PPE). Furan was removed quantitatively in solution under relatively mild thermal conditions via cycloreversion. The resulting unmasked polymer is functionalized with a compound containing a thiol or diene [36].

Recently, our research group has disclosed a novel strategy for the synthesis of reactive polymers bearing maleimide groups as side chain units. Polymers bearing protected maleimide groups as side chain substituents were prepared from a novel methacrylate based reactive monomer [25]. The reactive maleimide units were then activated via rDA reaction to enable post-functionalization of the formed polymer with thiol containing molecules. (Figure 1.14)

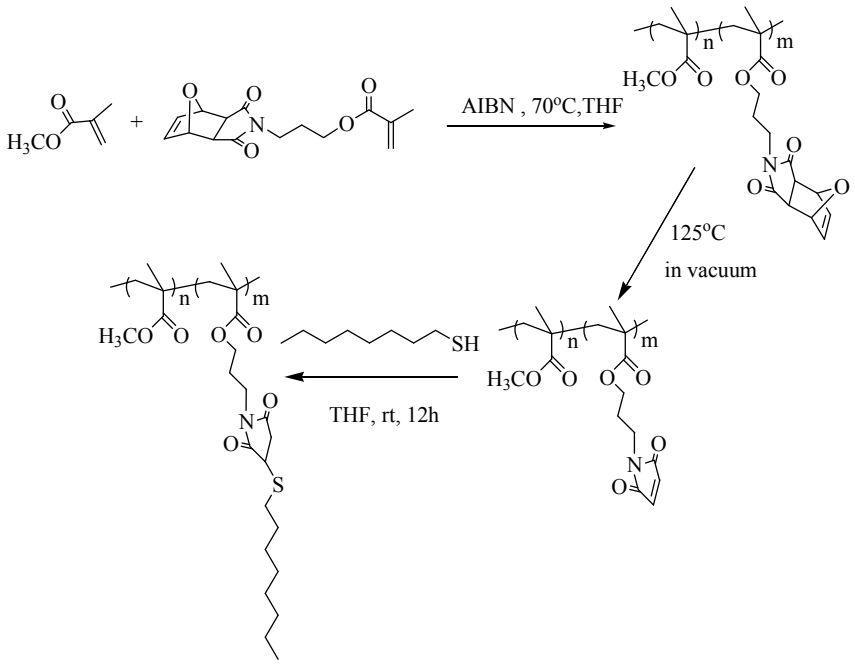


Figure 1.14. Synthesis of maleimide based reactive copolymer

2. AIM OF THE STUDY

2.1. Water Soluble Thiol Reactive Polymeric Supports

Water soluble biocompatible polymeric supports are instrumental in design and development of novel drug delivery platforms. Thiol reactive polymers are attractive candidates since thiol-ene type conjugations can allow attachment of thiol containing drug molecules and peptide units onto polymer backbone. Herein, water soluble poly (ethylene glycol) methacrylate based monoblock and diblock copolymers that contain thiol reactive maleimide side chains have been synthesized using the Atom Transfer Radical Polymerization method. Maleimide functionality is masked via Diels-Alder reaction during the polymerization and is activated afterwards by means of retro Diels-Alder cycloreversion reaction. Efficiency of post-functionalization was evaluated by the quantification of conjugation of the tripeptide glutathione (Figure 2.1).

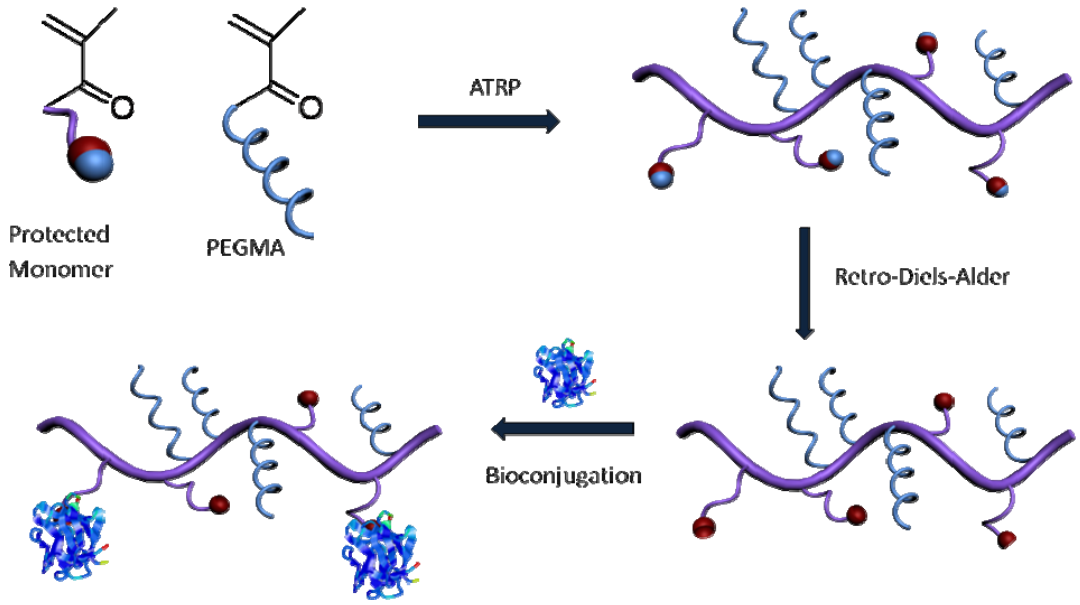


Figure 2.1. Synthetic approach towards maleimide based thiol reactive copolymers

2.2. Thiol Reactive Hydrogels for Bioimmobilization

Synthesis of thiol reactive poly (ethylene glycol) methacrylate based thiol reactive hydrogels are scaffolds that will provide a handle for controlled covalent biomolecular immobilization. Using a protection-deprotection strategy for incorporation of the maleimide functional group during gelation can be achieved. Copolymerization of a furan protected maleimide containing methacrylate monomer allows direct incorporation of latent maleimide groups within the hydrogel matrix. Thermal deprotection of the maleimide groups in some of the monomer results in the formation of an *in situ* crosslinker that leads to gelation without the need of additional crosslinker molecules. After gelation, protected maleimide groups are activated to their reactive forms via retro-Diels-Alder reaction. The swelling behavior and functional group density of maleimide units in the hydrogels can be modulated by tuning the feed ratio of monomers and gelation temperature. The extent of available maleimide groups within the hydrogels was investigated using thermogravimetric analysis. Successful covalent functionalization of the hydrogel was investigated using fluorescent labeling with thiol containing dye molecules and enzymes (Figure 2.2).

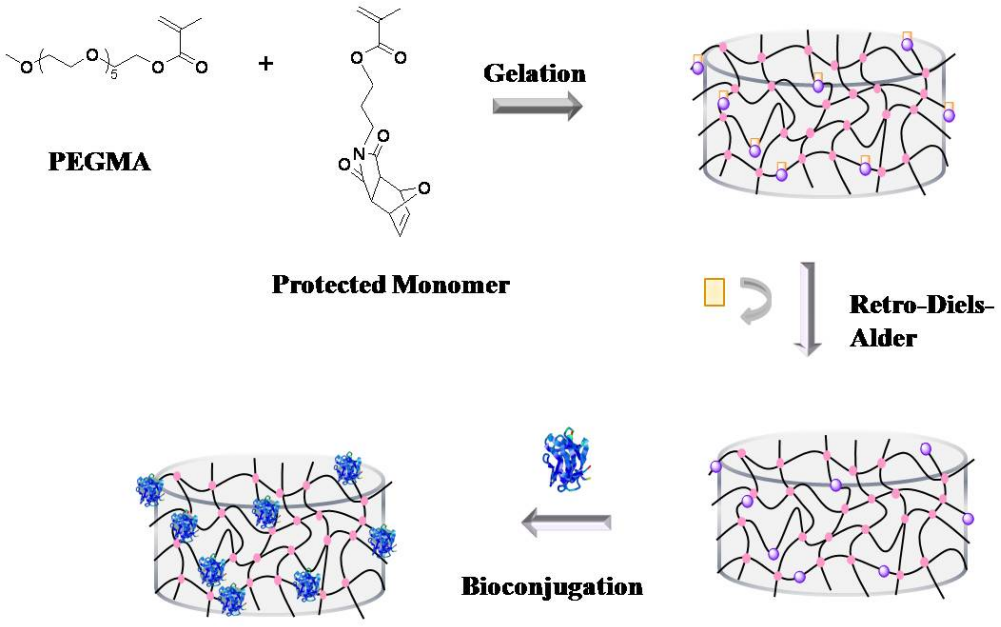


Figure 2.2. Synthetic approach towards maleimide based thiol reactive hydrogels

3. RESULTS AND DISCUSSION

3.1. Thiol Reactive Copolymers

The latent reactive maleimide monomer is copolymerized with poly (ethylene glycol) methacrylate via atom transfer radical polymerization, namely ATRP. The masked maleimide moeity is converted to the reactive maleimide functionality via retro Diels-Alder (rDA) reaction. In order to prove the efficiency of maleimide groups in thiol addition, reactive copolymer is functionalized with glutathione.

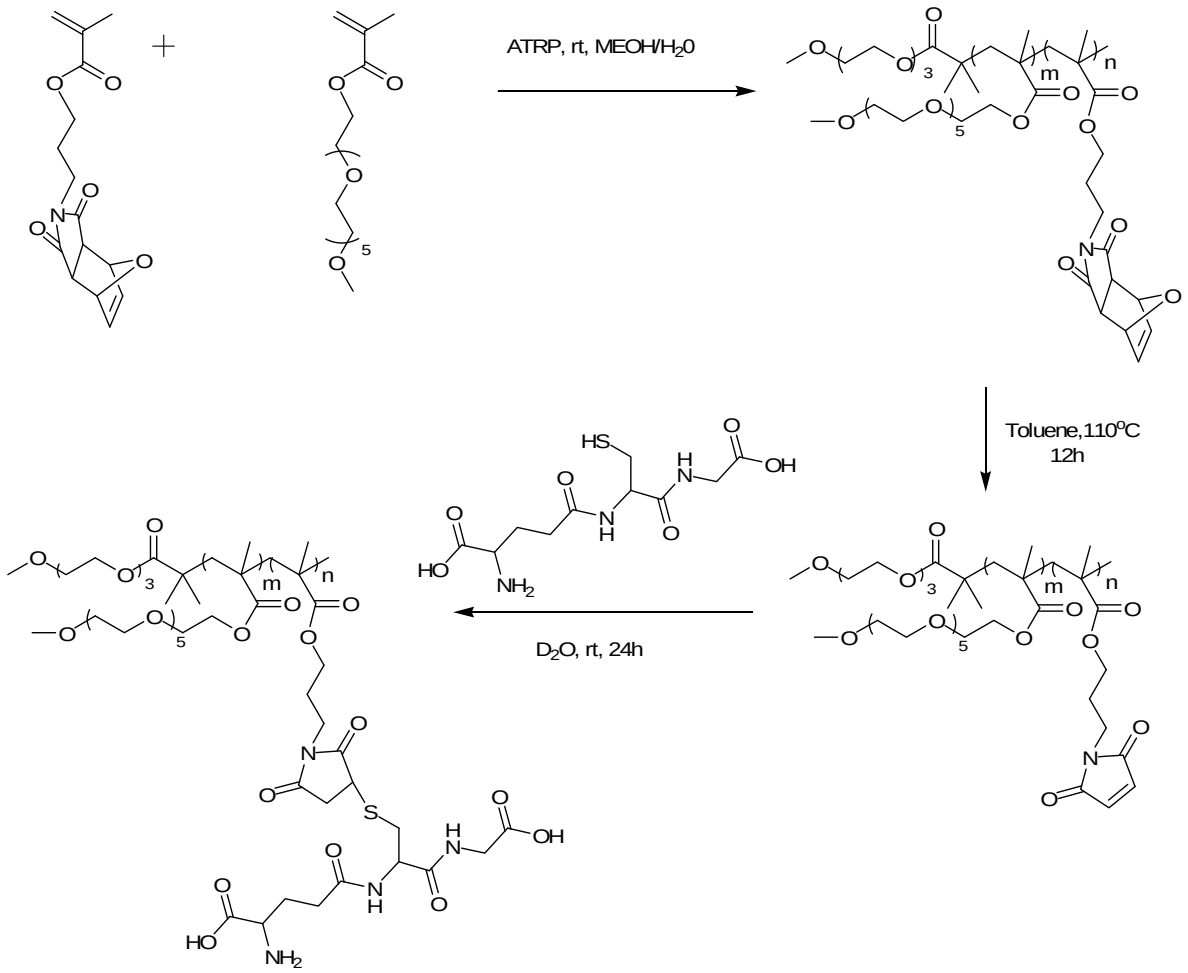


Figure 3.1. General scheme for thiol reactive polymers

It is crucial to use polymers with narrow polydisperties and known architecture for biological applications. To be able to have control over molecular weight distribution ATRP technique is used in polymerizations. ATRP is a controlled living polymerization method which is initiated by an alkyl halide (R-X) and catalyzed by a transition metal complex, such as CuX/bpy. In this study, triethylene glycol initiator is used as the alkyl halide initiator and CuBr/2,2'bipyridine as the catalyst system. Reaction time and solvent ratio is investigated for obtaining polymers with narrow polidispersities.

The aim is to obtain water soluble reactive copolymers, for this reason poly (ethylene glycol) monomethyl ether methacrylate is used as a comonomer since the maleimide monomer is not water soluble. Poly (ethylene glycol) (PEG) is chosen since it is water soluble and at the same time biocompatible and antibiofouling - meaning that it prevents bioadhesion. Antibiofouling property of the PEG stems from its protein resistivity due to steric repulsion between hydrated PEG chains and proteins.

In ATRP method, numerous combinations of solvent and temperature system can be used. In this study, it is aimed to polymerize monomers in presence of water. Since the latent reactive maleimide monomer is insoluble in water, a mixture of water and methanol is used as solvent system. In presence of water side reactions such as nucleophilic substitution of water to halide initiator or elimination of hydrogen halide may take place at elevated temperatures. For this reason reactions are carried out at room temperature.

Table 3.1 shows a summary of different polymerization conditions that were investigated and the results obtained. In all the polymerizations listed monomer: initiator:bpy:CuBr 100:1:2:1 ratio has been used. It has been observed that the polymerization is very fast and prolonged reaction times lead to broadening in molecular weight distribution, whereas no significant change occured in the average molecular weight.

In order to optimize the polymerization conditions PEGMEMA:Maleimide 4:1 ratio is used in several polymerizations. 6:1 dilution ratio is observed to be the most suitable solvent ratio since higher concentrations led to bimodal polymers and lower concentrations resulted in

low conversion. Methanol and water is used in equal amount for polymers containing less amount of maleimide eg. P3 and P4. Since the maleimide monomer is not soluble in water when the ratio of maleimide monomer increased 1:1 methanol: water system was not well suited. Methanol ratio is increased to solubilize the maleimide monomer. With 5:1 methanol: water ratio polymers with lower polydispersities were obtained (Table 3.1., P1,P2).

Table 3.1. Synthesis of latent-reactive monomer containing random copolymers

Polymers	PEGMEMA: Maleimide	Dilution a) Solvent:monomer b) MeOH: H_2O	Time	Conversion	Mn	Mw/Mn
P1	1:1	a)6:1 b)5:1	30 min	52%	8200	1.18
P2	2:1	a)6:1 b)5:1	30 min	55%	10900	1.21
P3	4:1	a)6:1 b)1:1	15 min	82%	15600	1.18
P4	8:1	a)6:1 b)1:1	15 min	86%	24500	1.31
P6	4:1	a)12:1 b)1:1	15 min	17%	11900	1.19
P7	4:1	a)6:1 b)1:1	6h	93%	18200	1.64
P8	4:1	a)6:1 b)1:1	4h	94%	18000	1.59
P9	4:1	a)6:1 b)1:1	2h	84%	16100	1.50
P10	4:1	a)6:1 b)1:1	1h	86%	18400	1.53

Polymerization of the monomer should result in disappearance of the proton resonances from the acrylate unit at 6.11 ppm (s,1H, CH_2=C) and 5.55 ppm (m, 1H, CH_2=C) (Figure 3.2., peak a & b, respectively) since unreacted monomers were eliminated by precipitating the polymer solution in diethyl ether. The composition of the copolymers could be easily determined from the integration of the ^{1}H NMR spectra. The ratio of area under the peak 6.51 ppm (peak a, Figure 3.3.) corresponding to the alkene protons of the monomer to the area

under the methyl ether group of PEGMEMA at 3.35 ppm (peak d, Figure 3.3.) was used in the determinations of copolymer compositions.

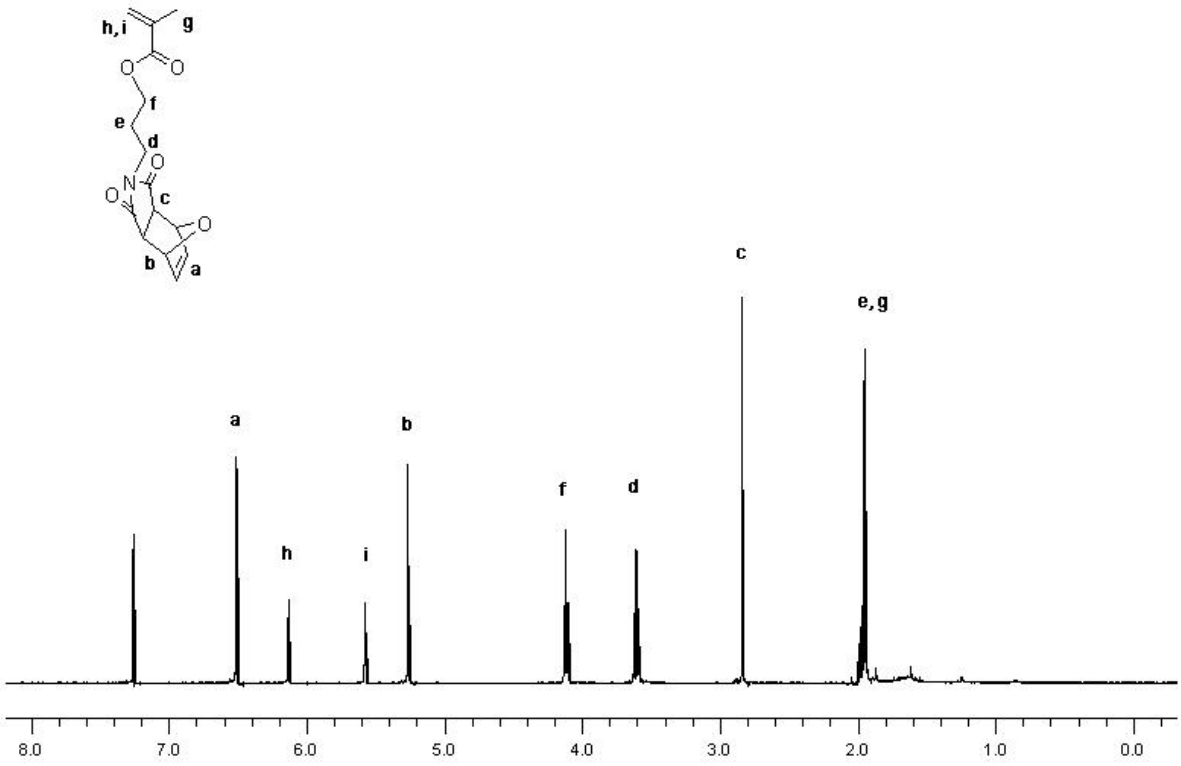


Figure 3.2. ^{1}H NMR of latent reactive monomer

3.1.1. Activation of the Polymer

The polymers were converted to their reactive form using the retro-Diels-Alder reaction. The polymers were heated for 12h at 110 ^{o}C using toluene as the solvent. This resulted in complete cycloreversion of the furan-maleimide adducts to afford thiol reactive polymers bearing maleimide groups in the side chain. ^{1}H NMR analysis proved that the cycloreversion was almost quantitative (Figure 3.4). Appearance of a new peak at 6.75 ppm (peak a, Figure 3.4.), accompanied by disappearance of peaks at 5.25 and 6.51 ppm (Figure 3.3., peaks b&a respectively) corresponding to the oxabicyclic moiety shows successful cycloreversion. GPC

analysis proved that there was no detrimental effect on the polymer as confirmed by presence of a monomodal peak with low polydispersity.

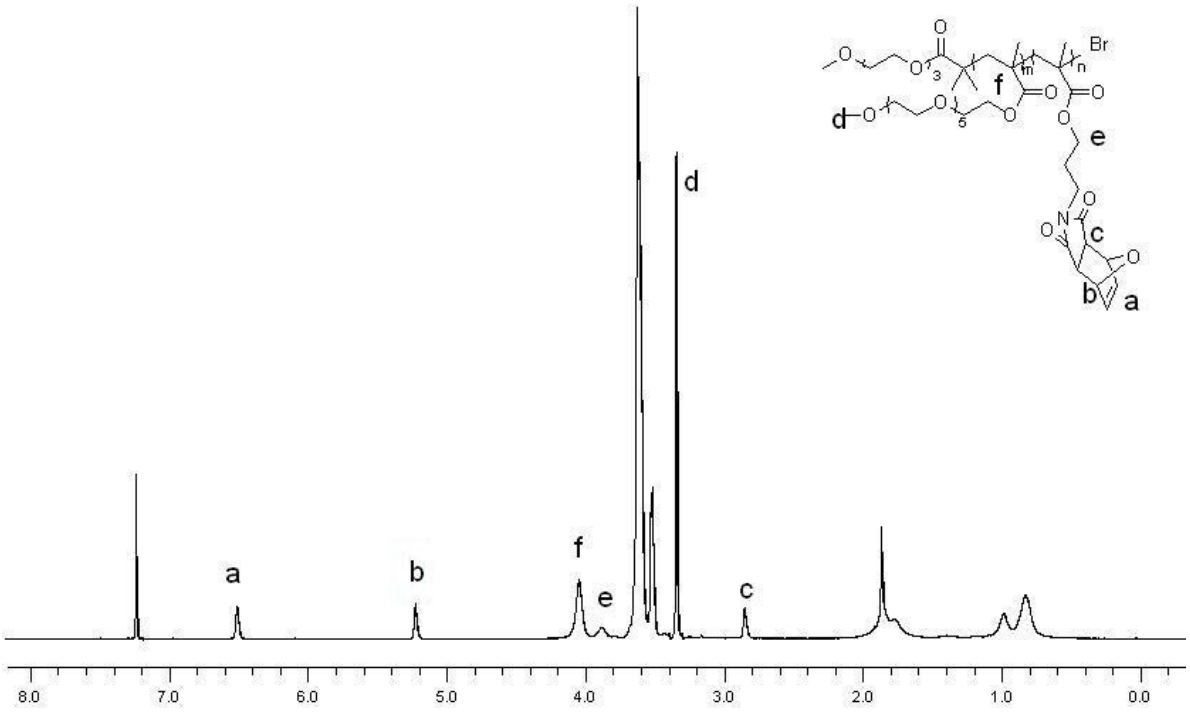


Figure 3.3. ^{1}H NMR of the polymer 3

Another way of monitoring the efficiency of the cycloreversion reaction is thermogravimetric analysis. After the rDA there has to be no significant weight loss between 90-180°C. As expected, no weight loss in that region was observed upon analysis of the polymer 3 after retro (P5) obtained after the cycloreversion step, due to lack of any furan adducts in the side chains (Figure 3.5).

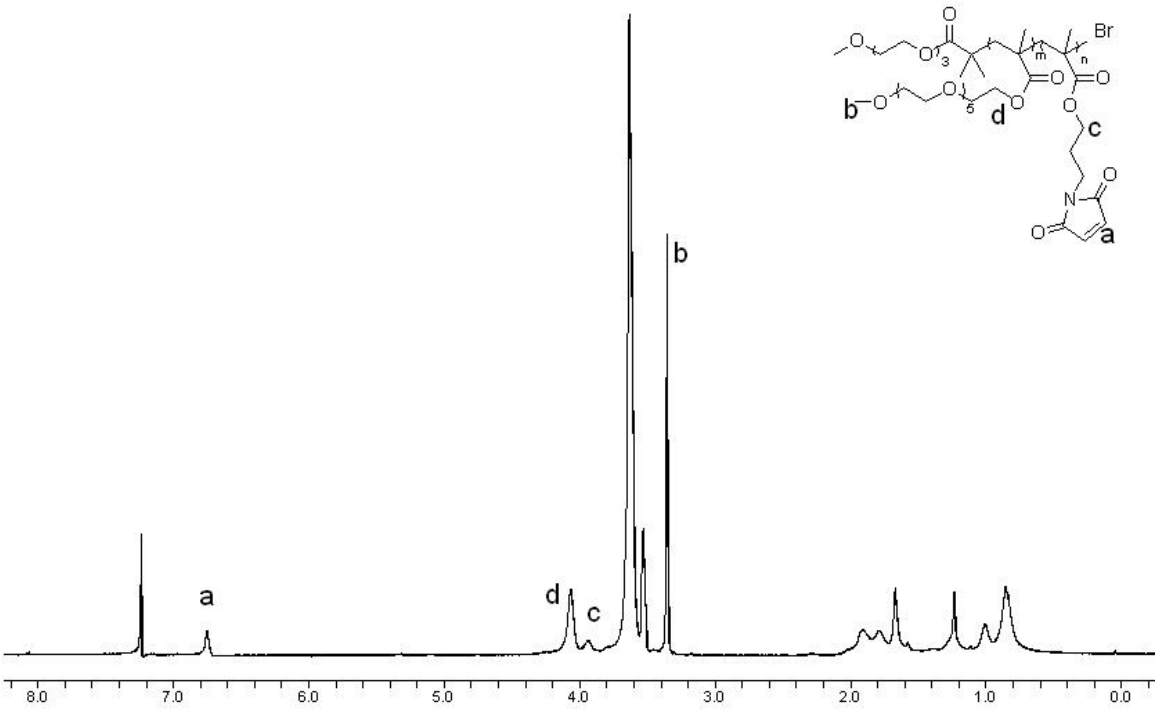


Figure 3.4. ^{1}H NMR of activated polymer

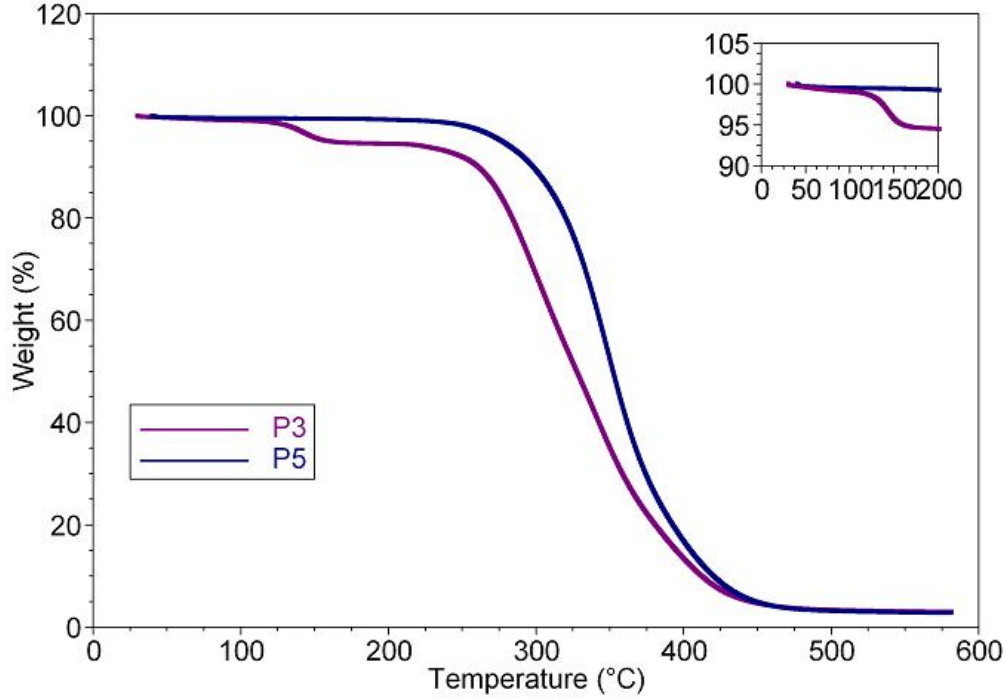


Figure 3.5. Thermogravimetric analysis of polymers before and after cycloreversion

Thermogravimetric analysis (TGA) was used to determine the thermal stability of the copolymers and investigate the activation of the maleimide groups via loss of furan during rDA reaction. Thermogravimetric analysis (TGA) of the polymers **P(1 – 4)** showed a weight loss starting at 90 ^{o} C (Figure 3.6.). A consistent increase in weight loss of the polymers was observed upon increasing the amount of furan based monomer. According to the TGA analysis the observed weight losses were 13.9%, 8.6%, 4.5% and 2.8% for polymers **P1**, **P2**, **P3** and **P4** respectively. These were higher than the expected weight losses (11.4%, 7.6%, 4.6% and 2.5%). Similar observations have been reported before and the discrepancies have been attributed to the broad range of local environment around the cycloadducts [36].

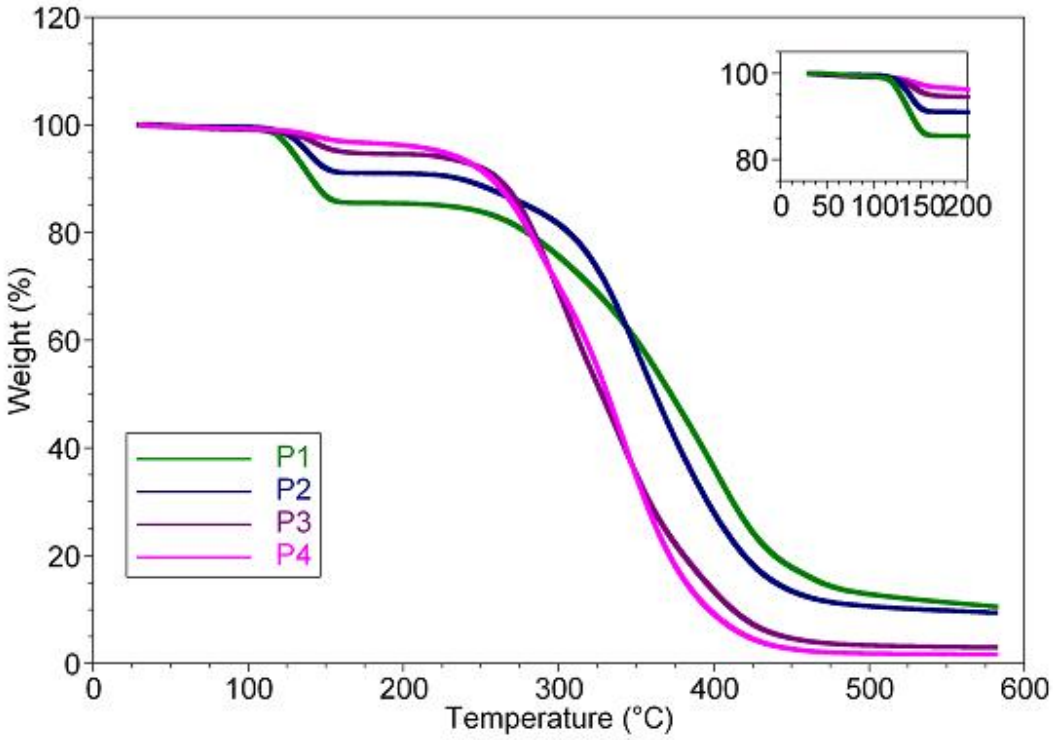


Figure 3.6. Thermogravimetric analysis of polymers

3.1.2. Functionalization with Glutathione

Polymer **P3** was functionalized with glutathione to prove the reactivity of the polymers towards thiols. The disapperance of the peak at 6.75 ppm (Figure 3.7B) corresponding to the double bond of the maleimide functionality along with appearance of expected peaks showed the successful addition of glutathione (Figure 3.7A). The Michael addition reaction between thiols and activated alkenes takes place efficiently under mild conditions in aqueous media. To demonstrate the efficiency of the reaction **P3** is reacted with 0.3, 0.6 and 1.0 equivalent of glutathione and ^{1}H NMR and CHNS data showed quantitative functionalization in each step. This indicates that without the presence of different functional groups on the polymer side chain, orthogonal functionalization can be achieved.

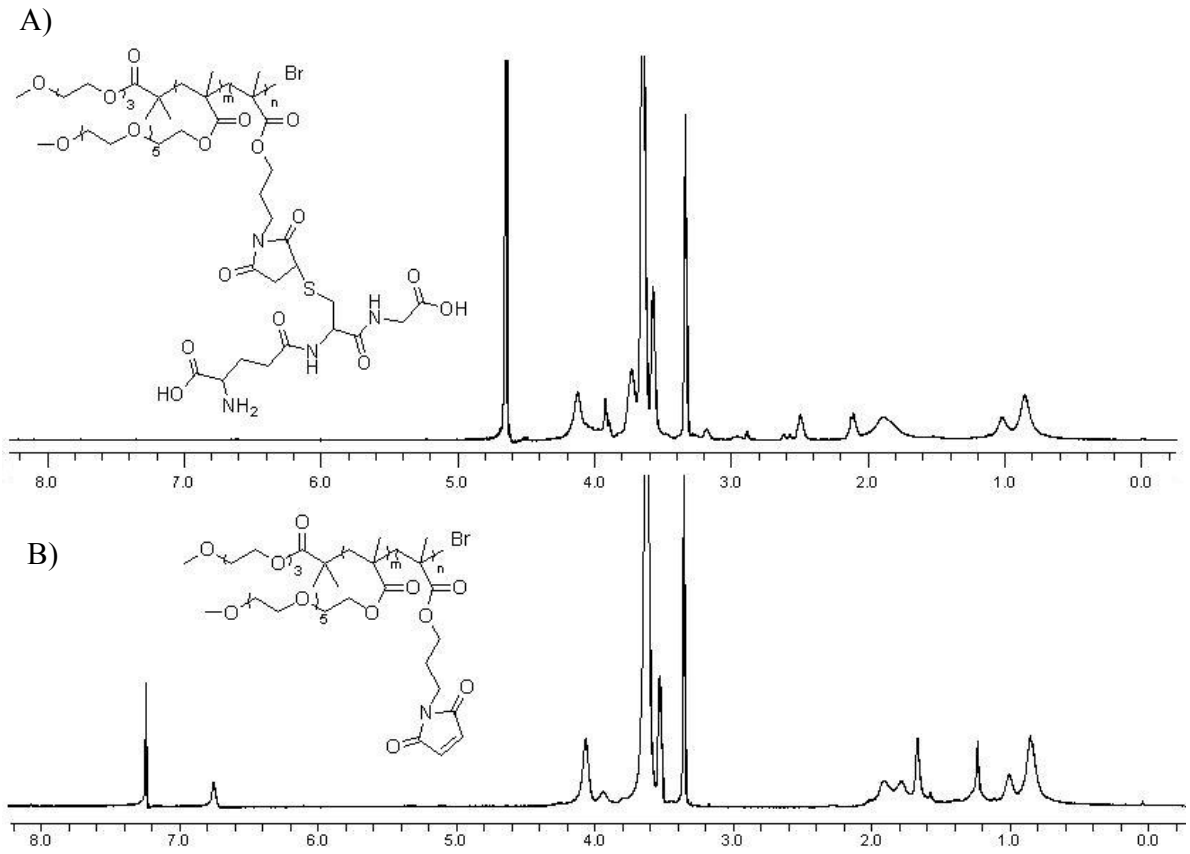


Figure 3.7 A) ^{1}H NMR of functionalized polymer B) ^{1}H NMR of activated polymer

In the spectra, from the integration ratio of the maleimide proton resonance at 6.75 ppm and $-OCH_3$ proton resonance of poly(ethylene glycol) monomer at 3.35 ppm, the amount of reacted maleimide groups can be calculated (Figure 3.6). The integration of the above mentioned proton resonances demonstrated highly efficient conjugation (Figure A.3.). In the elemental analysis, increase in the sulphur and nitrogen content was observed as the amount of conjugated glutathione increased. The increase in the sulphur content in the elemental analysis was found to be directly proportional to the amount of the conjugated glutathione.

Table 3.2. Results of elemental analysis

Polymers	Nitrogen	Carbon	Hydrogen	Sulphur
Polymer 3	1.35	55.49	8.29	0
Polymer conjugated to 0.3 eq glutathione	1.94	54.07	7.95	0.26
Polymer conjugated to 0.6 eq glutathione	2.43	53.26	7.77	0.51
Polymer conjugated to 1 eq glutathione	3.44	51.72	7.58	1.16

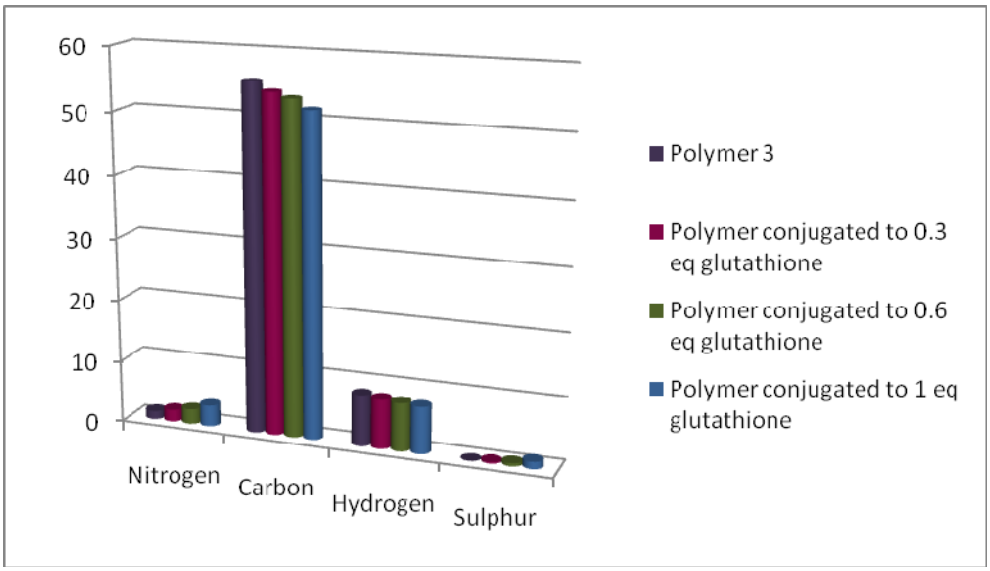


Figure 3.8. CHNS results

3.1.3. Diblock Copolymers

The synthesis of copolymers was extended to synthesis of diblock copolymers using a poly (ethylene glycol) macroinitiator (Figure 3.9). Block copolymers are hybrid macromolecules that can be organized into one-, two-, or three-dimensional periodic nanostructures according to their compositions. They can self-assemble to form a variety of nanoscale morphologies including spheres, rods, lamellae, vesicle tubules, cylinders, and large compound vesicles, micelles, or rod micelles.

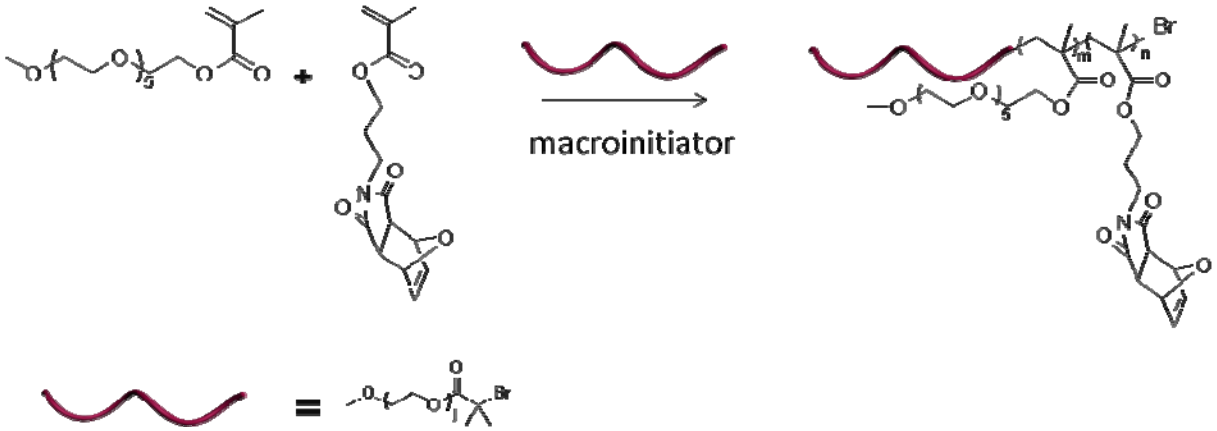


Figure 3.9. General scheme for diblock polymer synthesis

Two different types of block copolymers were synthesized using 2K and 5K PEG macroinitiators. The macroinitiators and polymers were characterized using gel permeation chromatography. GPC analysis showed the successful synthesis of macroinitiators, as confirmed by presence of monomodal peaks with polydispersities close to 1 as can be seen in Figure 3.10.

It has been observed that solvent ratio had a prevailing influence on the polydispersity. The molecular weight and polydispersities obtained by changing the ratio between MeOH and water is summarized in Table 3.3. In all the polymerizations listed in Table 3.3, PEGMEMA:Maleimide 4:1 ratio was used and polymerization time was 30 minutes. **P11** and

P12 polymers were synthesized using 5K PEG macroinitiator whereas **P13** and **P14** polymers were synthesized by 2K PEG macroinitiator.

Table 3.3. Diblock Polymers

Polymers	Initiator	Dilution a) solvent : monomer b) MeOH : H_2O	Conversion	Mn	Mw/Mn
P 11	5K	a) 6:1 b) 1:1	%86	22766	1.77
P 12	5K	a) 6:1 b) 5:1	%83	23469	1.34
P 13	2K	a) 6:1 b) 1:1	%89	20100	2.00
P 14	2K	a) 6:1 b) 5:1	%66	12587	1.38

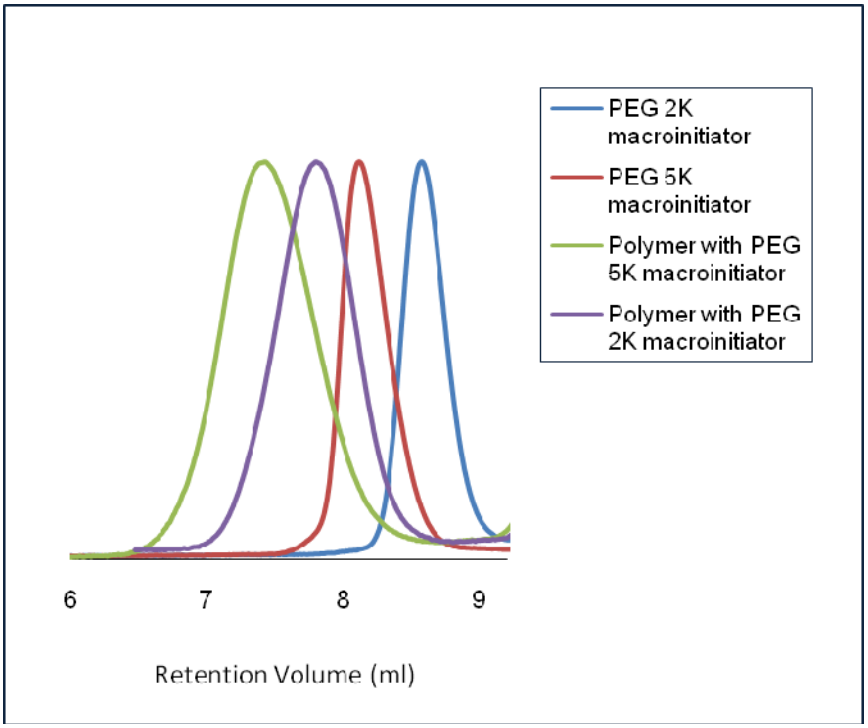


Figure 3.10. GPC analysis of diblock polymers **P12** (5K),**P14** (2K)and macroinitiators

3.2. Thiol Reactive Hydrogels

Copolymerization of the latent-reactive maleimide monomer with PEGMEMA via free radical polymerization at high concentrations led to hydrogel formation due to activation of the maleimide double bond.(Figure 3.11.) By changing the maleimide monomer ratio, PEG chain length and reaction temperature, a library of hydrogels have been prepared. Functionalization of the hydrogels is demonstrated by conjugation of a fluorescent dye and an enzyme.

At high concentrations, hydrogels were obtained by copolymerization of monomer **I** and PEGMEMA via 2,2'-azobisisobutyronitrile (AIBN) initiated free radical polymerization within 30 minutes. The polymerization concentration was found to be a very important factor in the formation of the hydrogels. We have recently demonstrated that the copolymerization of monomer **I** along with methyl methacrylate in THF at 70°C leads to soluble copolymers. In dilute solutions (< 0.14g/mL), no crosslinking was observed even after 24 hours. Hydrogel synthesis is performed using concentrated solutions (1.0g/mL). Thus, obtained gels remain insoluble in various solvents at high temperatures.

It is proposed that the observed crosslinking during the formation of these covalently functionalizable gels occurs due to the *in situ* retro Diels-Alder reaction of the furan protected monomer. The release of furan produces a monomer containing two reactive double bonds. This bifunctional fragment acts as a crosslinker. As a control experiment, gelation reaction was attempted using monomer **II** which is the hydrogenated version of monomer **I.** Since it is lacking the double bond in the bicyclic fragment it is incapable of undergoing a retro Diels-Alder cycloreversion to generate the crosslinker. The characterization of the monomer II is done by ^{1}H NMR spectroscopy. The disappearance of alkene peak at 6.51 ppm (Figure 3.12., peak a) of the oxabicyclic moiety and presence of new alkane peaks between 1.55-1.85 ppm (Figure 3.13., peak a) proved the hydrogenation and synthesis of monomer II.

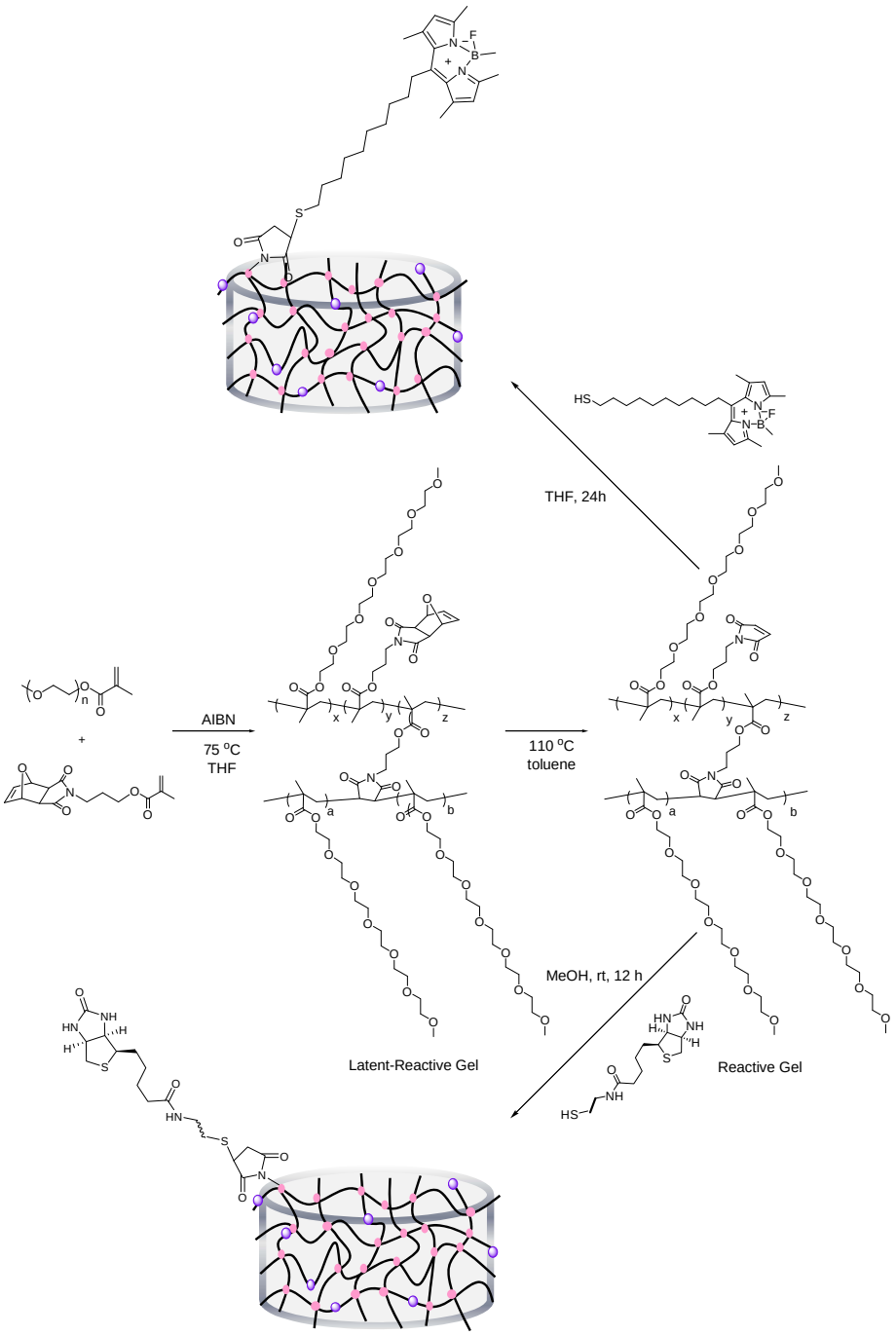


Figure 3.11. General scheme for the synthesis and functionalization of hydrogels

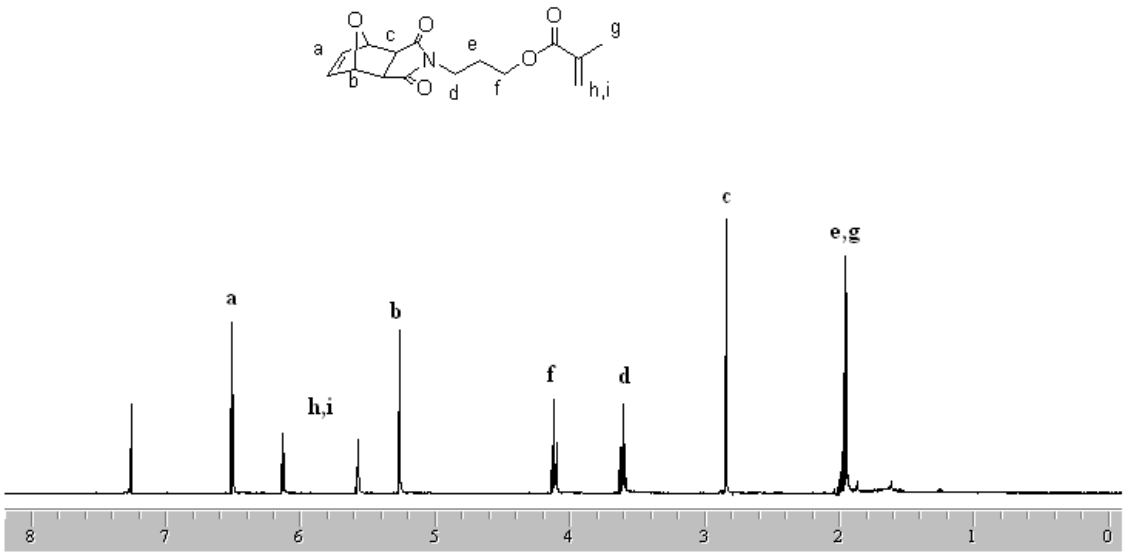


Figure 3.12. ^{1}H NMR of Monomer I

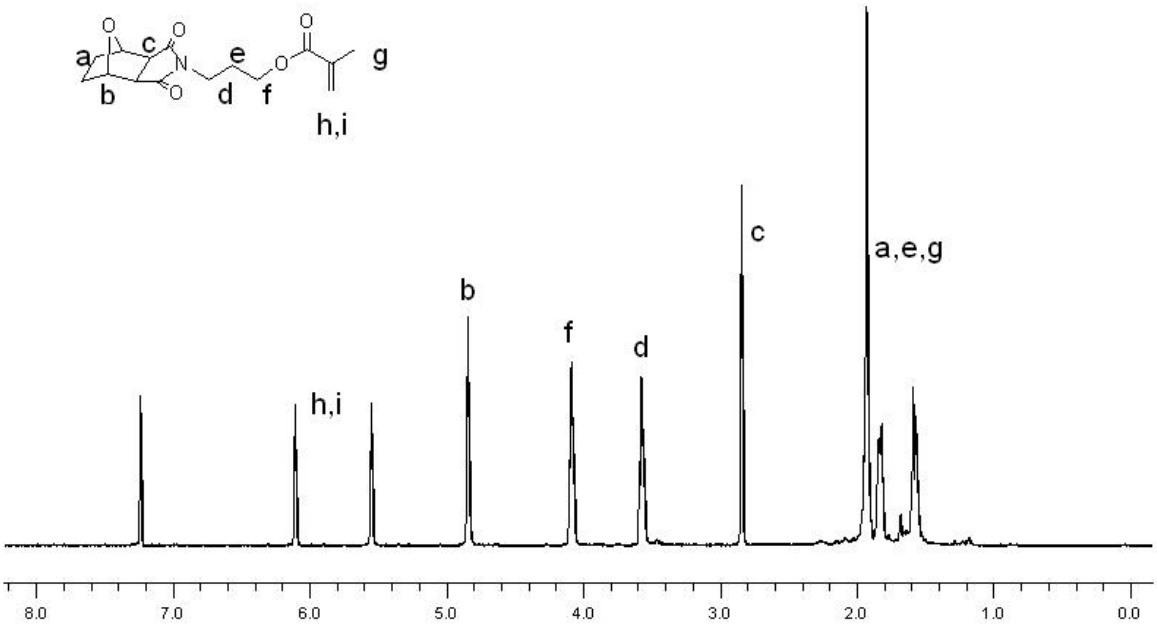


Figure 3.13. ^{1}H NMR of Monomer II

Several reactions are performed using different amount of monomer II along with PEGMEMA at 75 and 90°C. As expected, no gels were obtained upon using this monomer.

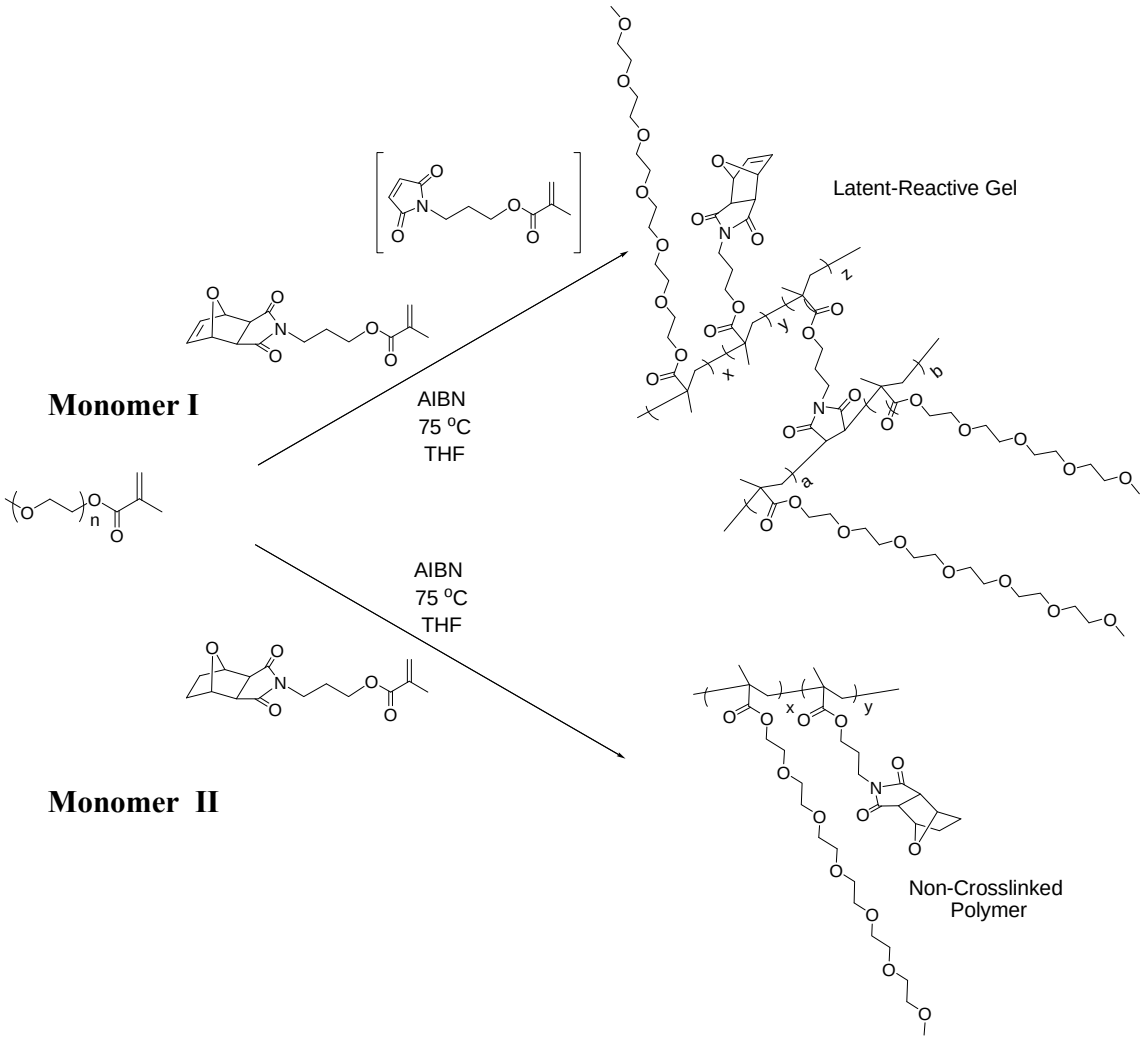


Figure 3.14. Proposed mechanism of Gel Formation

The physical and chemical characteristics of the hydrogels are highly dependent on the experimental conditions such as feed ratio, initiator amount, temperature, and post gelation

treatment. In order to investigate the role of each of these parameters a variety of hydrogels were synthesized. These results are summarized in Table 3.4 and the observations are discussed thereafter.

Table 3.4. Properties of hydrogels

Item	Hydrogels	Temperature	Mwt of PEGMEMA	Monomer I : PEGMEMA	Furan (%) observed[a]	Furan (%) theoretical[b]	Gel Content (%)
1	**H1**	75° C	300	1:10	2.21%	2.06%	96.%
2	**H2**	75° C	300	2:10	3.54%	3.79%	93.1%
3	**H3**	75° C	300	4:10	5.11%	6.51%	96.2%
4	**H4**	90° C	300	1:10	1.59%	2.06%	90.4%
5	**H5**	90° C	300	2:10	2.37%	3.79%	92.3%
6	**H6**	90° C	300	4:10	3.22%	6.51%	94.6%
7	**H7**	75° C	750	4:10	2.81%	3.14%	95.7%
8	**H8**	75° C	1100	4:10	2.26%	2.23%	94.1%

a: Measured by TGA

b: Calculated theoretically

3.2.1. Effect of Feed Ratio on the Functional Group in the Gel

One can expect that an increase in the amount of protected maleimide monomer in the feed ratio will result in formation of gels with higher content of protected maleimide units under the same experimental conditions. The amount of protected maleimide units within the gel can be probed using thermogravimetric analysis (TGA). The weight loss between 60-180 ^{o}C corresponds to the removal of the furan molecules generated during the retro-Diels-Alder

reaction. As expected, an increase in the loss of furan moiety is observed for gels with higher content of latent reactive monomer (Table 3.4.Item 1-3 & 4-6). Thus, control over the extent of reactive functional groups within the gels can be achieved. Figure 3.14 shows the increase in the weight loss going from **H1** to **H3**. Same trend was obtained for the hydrogels prepared at higher temperature (**H4-H6**).

The more protected maleimide monomer is present, the higher is the crosslink density. The increase in the crosslink density can also be monitored by thermogravimetric analysis from the decomposition temperatures. As the crosslink density increases hydrogels become more stable and decompose at higher temperatures.

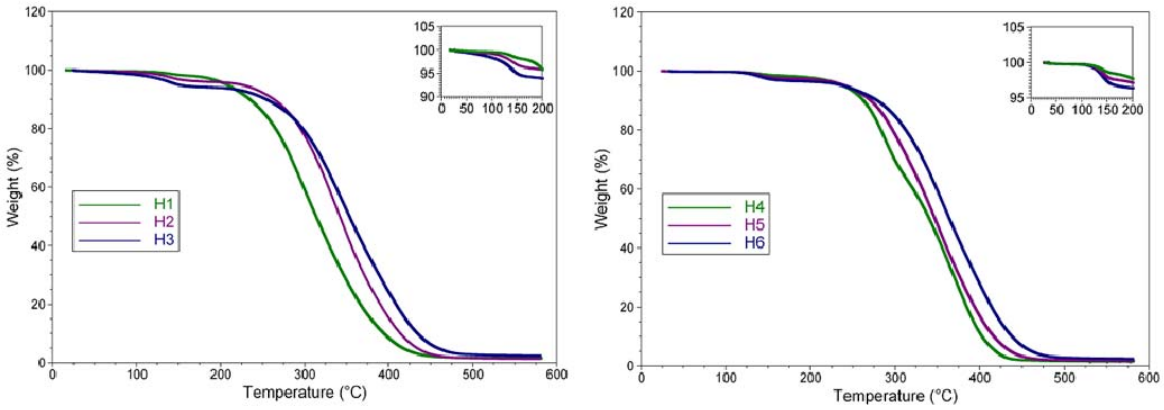


Figure 3.15. Thermogravimetric analysis effect of feed ratio

3.2.2. Effect of Temperature on Functional Group in the Gel

Increase in the reaction temperature should lead to more in situ fragmentation of the latent reactive monomer to produce more of the crosslinking reactions. This would result in decrease in the amount of protected maleimide units within the gel. Gels were prepared at different temperatures for the same feed ratio of the monomers to investigate the effect of temperature. As expected, the TGA analysis of the hydrogels revealed a decrease in the

amount of furan released as the temperature of gelation was increased. **H1** and **H3** possess a lower weight loss compared to **H4** and **H6** respectively (Figure 3.16).

Since the number of crosslinking reactions were higher with increasing temperature the hydrogels prepared at higher temperatures were more stable. **H1** and **H3** started to decompose at lower temperatures relative to **H4** and **H6,** respectively.

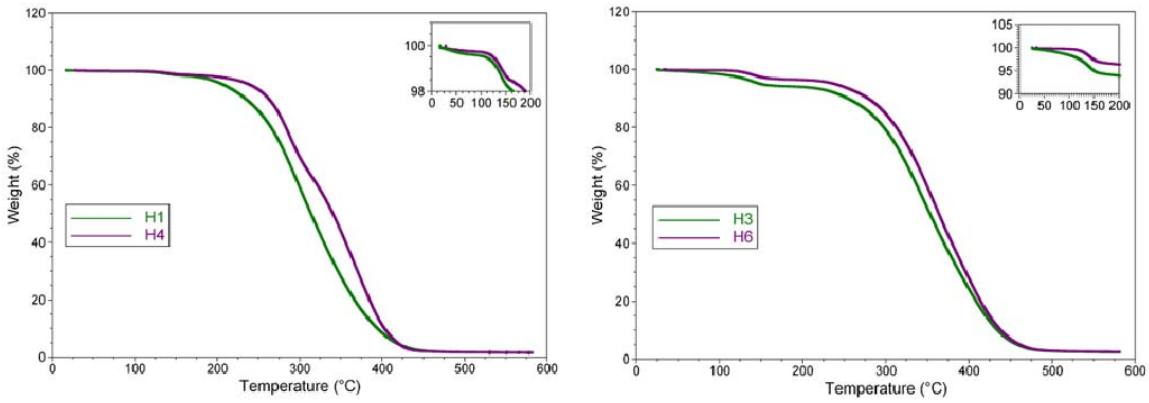


Figure 3.16. Thermogravimetric Analysis effect of temperature

3.2.3. Swelling Behavior of the Hydrogels

The swelling behavior of hydrogels is dependent on crosslink density and can be controlled by variation of amount of maleimide monomer and by changing the temperature. Also PEG chain length has a very dominant effect on the swelling ratio. Since PEG is a hydrophilic polymer, swelling ratio of the hydrogels increase with increasing PEG chain length (Figure 3.16c). As the maleimide monomer ratio increased, the hydrophobic nature of the hydrogels increased resulting in lower water uptake. The same trend was observed with increasing crosslink density.

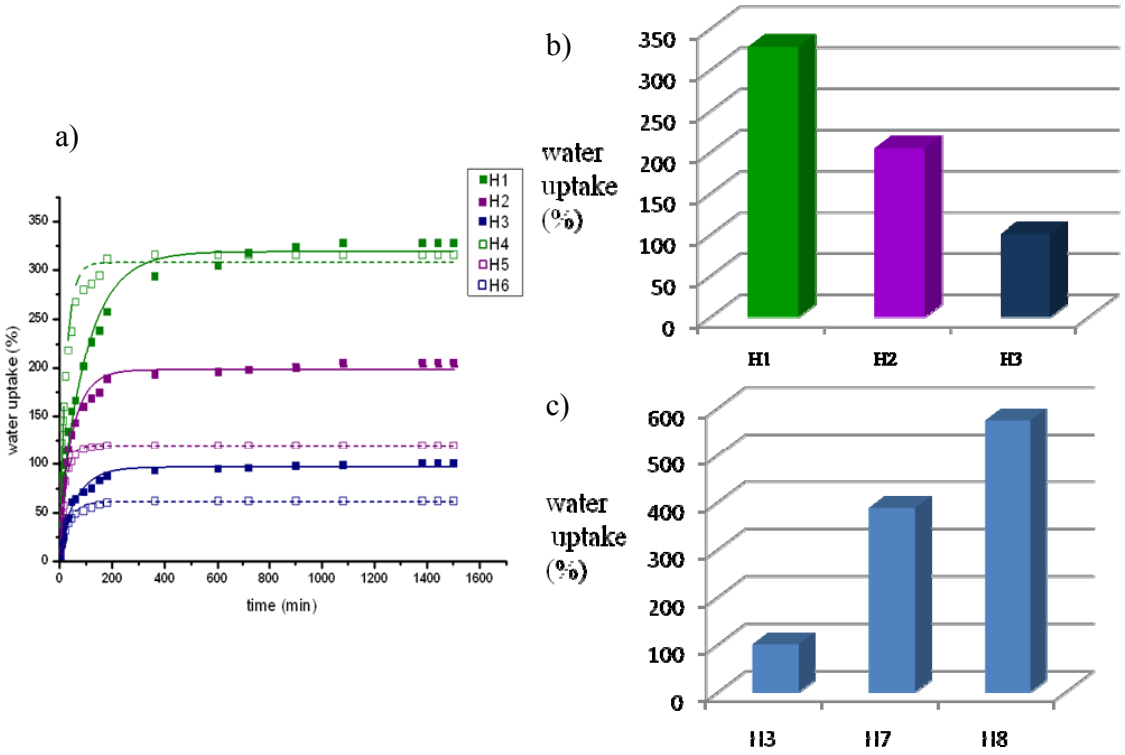


Figure 3.17. a) Swelling ratios of different Hydrogels b) Effect of maleimide ratio on Swelling c) Effect of PEG chain length on swelling

3.2.4. Functionalization Studies

To demonstrate the functionality of maleimide groups present on the hydrogels, several functionalization studies were performed. The hydrogels were reacted with thiol containing molecules via Michael addition reaction after the activation of the maleimide groups through rDA.

The efficiency of the rDA reaction is monitored by thermogravimetric analysis. As expected, no weight loss was observed between 60-180°C upon subjecting the hydrogel **H3** to the cycloreversion step, due to lack of any furan adducts in the side chains. (Figure 3.16).

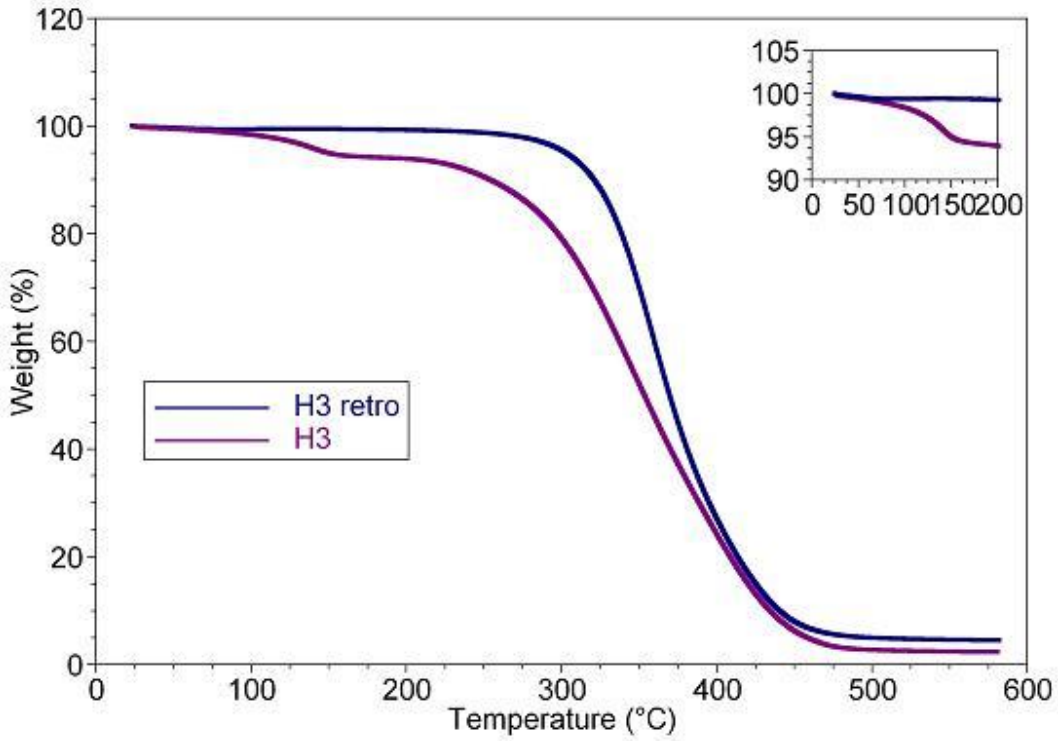


Figure 3.18. Activation of hydrogels

3.2.5.1. Functionalization with BODIPYC10SH: The hydrogels were reacted with a thiol containing fluorescent dye BodipyC10SH. (Figure 3.18) Bodipyc10SH was synthesized according to literature by Bizzotto *et al* [37]. After functionalization the hydrogels showed fluorescence and the control samples incubated with a solution of BODIPY-Br showed no fluorescence.

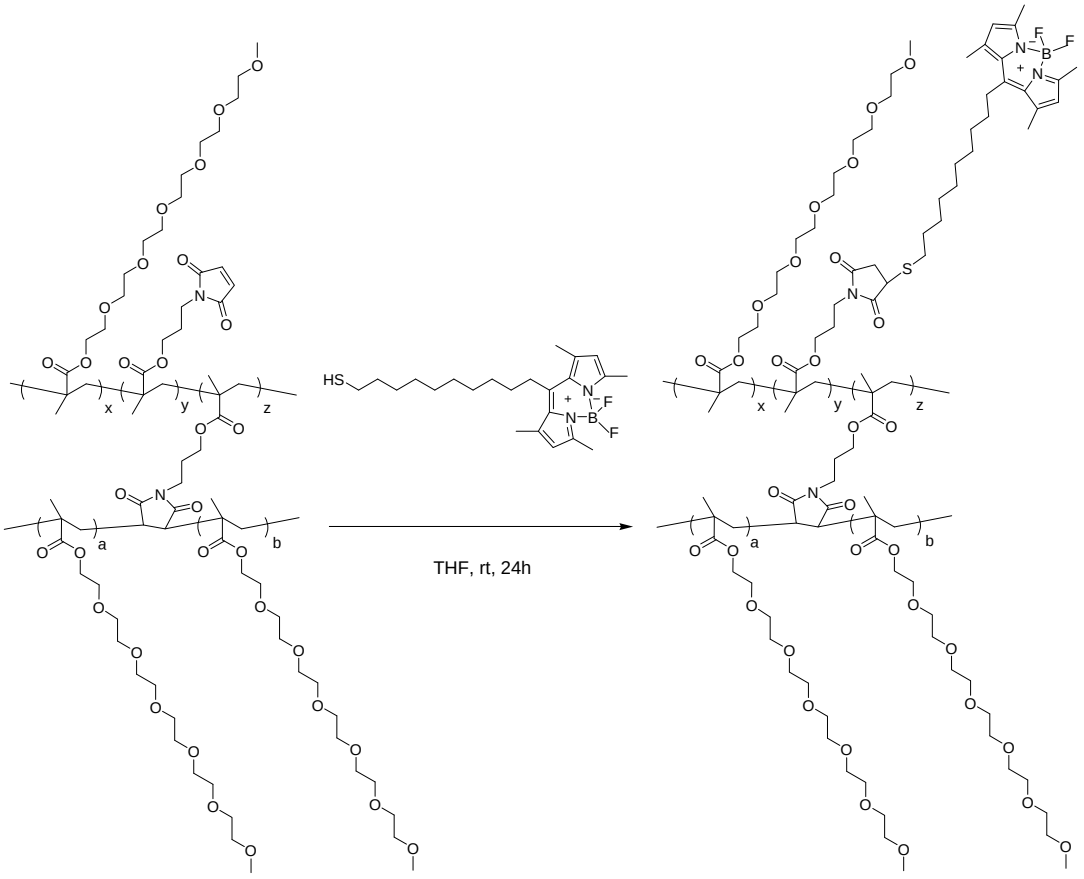


Figure 3.19.Conjugation with BODIPYCH10SH

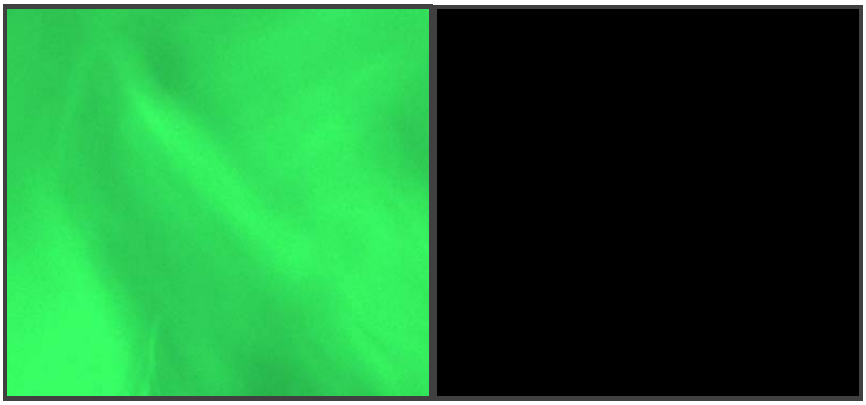


Figure 3.20. Fluorescence microscope images of H1 retro reacted with a) BodipySH, b)Bodipy Br

3.2.5.2. Controlled Streptavidin Immobilization: The potential of these gels to act as templates for bioimmobilization of enzymes in a controlled manner was evaluated. Thiol containing biotin was covalently attached to the gels and their availability for immobilization of streptavidin was investigated (Figure 3.20). Hydrogels **H1** and **H3** with different degree of maleimide groups were reacted with excess thiol containing biotin. After washing off excess biotin from the hydrogel to remove any unbound biotin, hydrogels were exposed to FITC labeled streptavidin. After washing off physiabsorbed streptavidin from the hydrogel, extent of immobilization was investigated using fluorescence microscopy. As expected gels containing more covalently bound biotin were able to immobilize more streptavidin (Figure 3.22a). Also there is a correlation between the amount of maleimide groups present and the amount of fluorescence intensity (Figure 3.22b). Maleimide containing gels which are not biotinylated were used as controls. These gels were exposed to FITC-streptavidin and washing protocols. Lack of fluorescence relative to biotinylated gels shows that the PEG matrix acts as an antibiofouling scaffold. Figure 3.22 shows the relative fluorescence of **H1**, **H3** and the control sample. **H3** possesses the brighter fluorescence and the control sample did not show fluorescence.

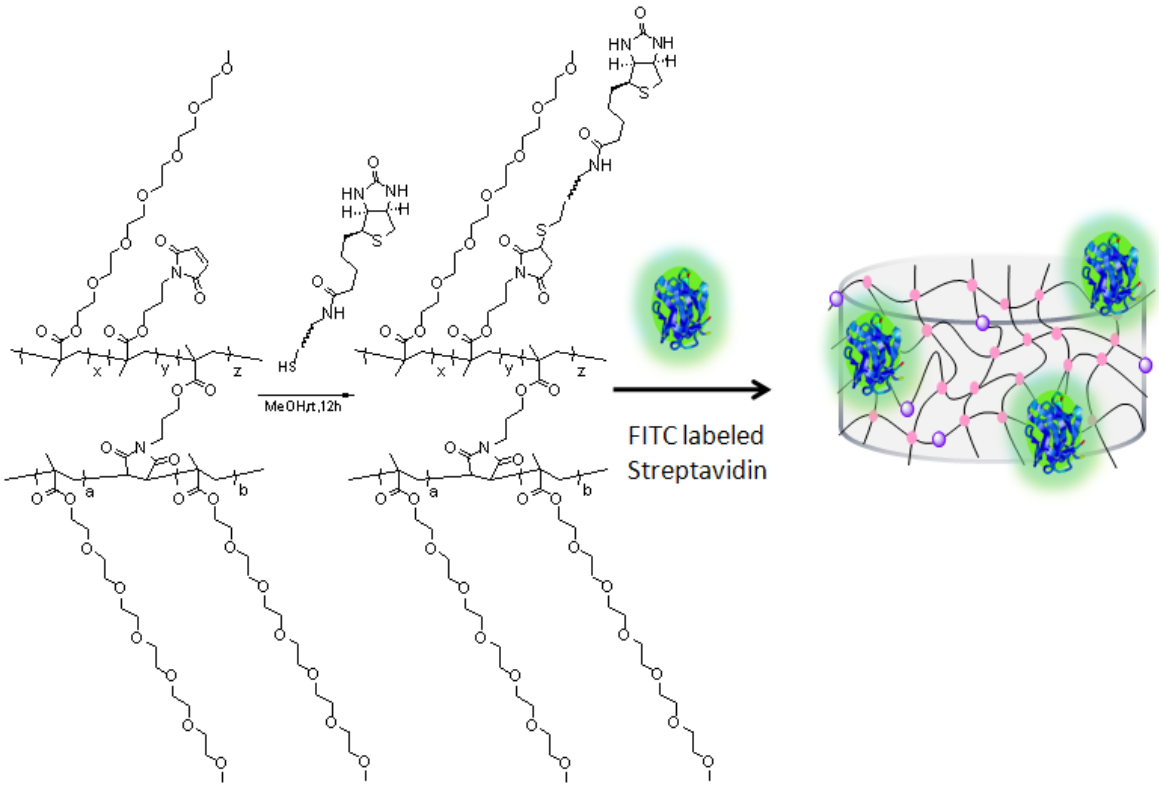


Figure 3.21. Streptavidin Immobilization

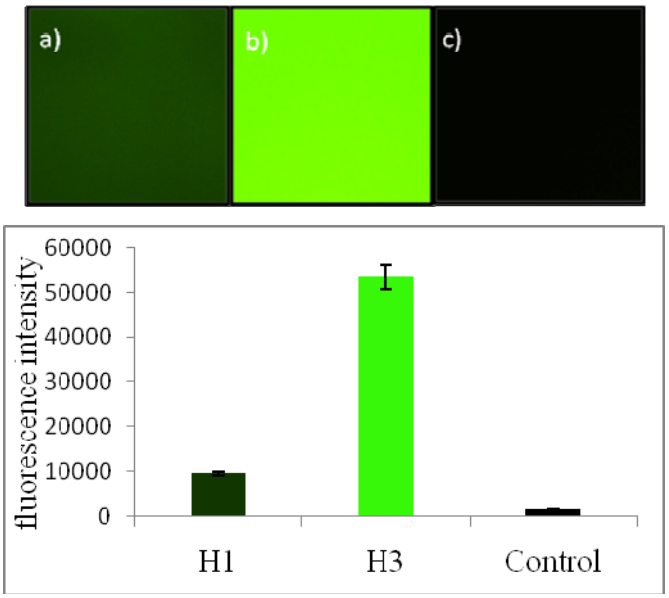


Figure 3.22. a) Fluorescence microscope images b) Calculated fluorescence intensities of biotinylated **H1**, **H3** and **H3** without biotinylation

3.2.6. Scanning Electron Microscopy

Hydrogel topography is investigated using scanning electron microscopy. The hydrogel samples were immersed in H_2O solution at room temperature to reach an equilibrium state. The swollen hydrogel samples were quickly frozen and further freeze-dried under vacuum until the solvent was sublimed. The freeze-dried samples were then examined by using a scanning electron microscope. It has been observed that the hydrogels showed a microporous structure.

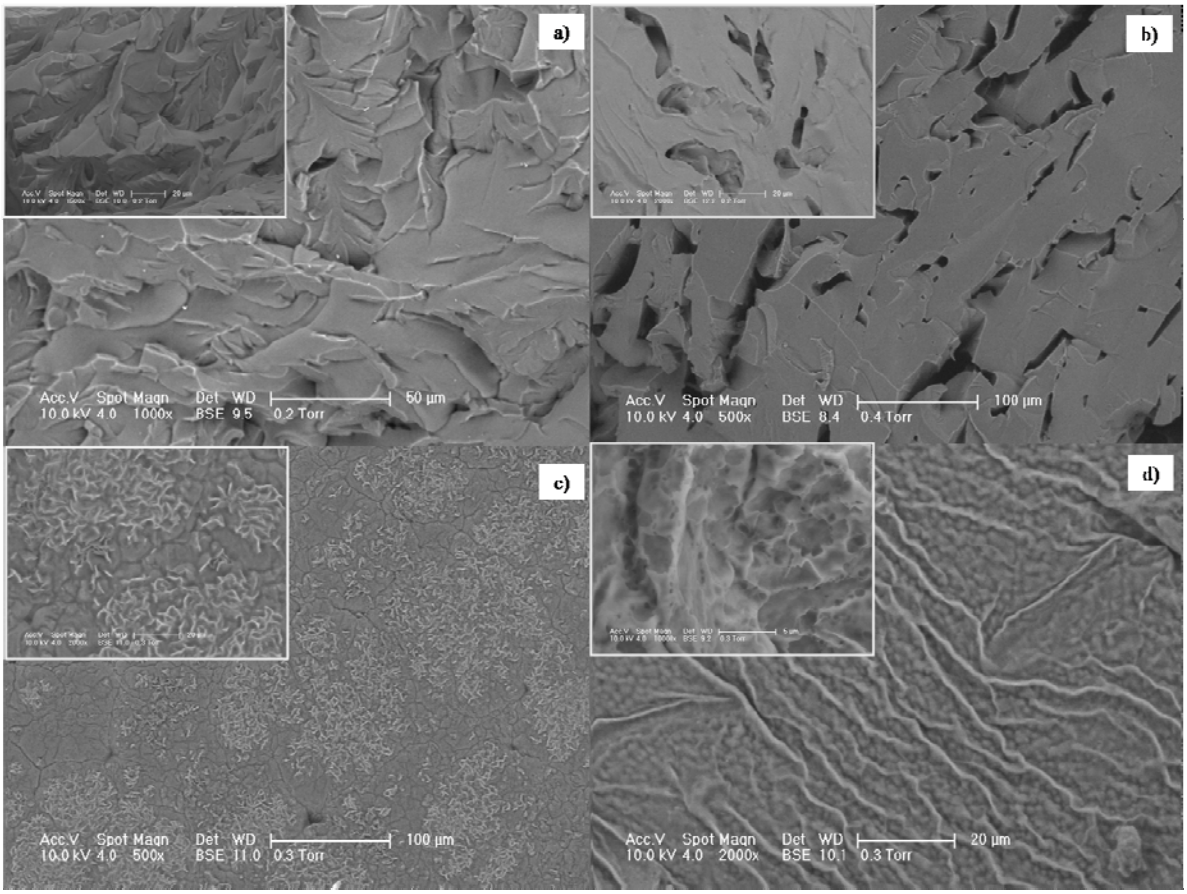


Figure 3.23. Scanning Microscope Images of a) **H4** b) **H3** c) **H7** d) **H8**

3.2.7. Thermoresponsive Property of The Hydrogels

It has been well documented in recent years that polymers containing pendent oligoethylene chains exhibit temperature stimuli behavior [38]. For the hydrogels synthesized in this study, thermoresponsive behavior was observed. Parent hydrogel was clear and transparent. Loss of transparency was observed upon warming the gel. This change was reversible as complete transparency was observed upon cooling of the gel. The LCST (lower critical solution temperature) of the hydrogels was measured by UV spectroscopy. Hydrogels were incubated in deionized water at room temperature overnight to swell and reach an equilibrium state. Transmittance of hydrogel in deionized water as a function of temperature was observed at 670 nm. The LCST was found to be 75 °C. Below this temperature hydrogels were clear and transparent where as above this temperature the hydrogels became opaque.

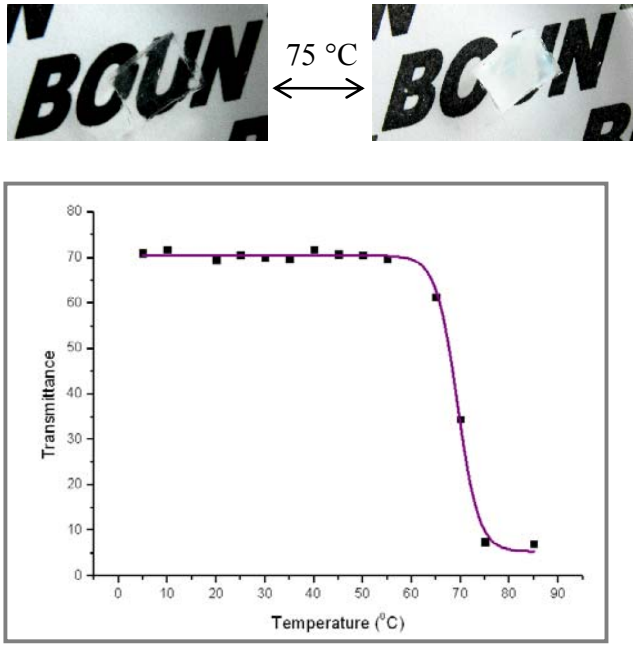


Figure 3.24. Lower critical solution temperature of H1

4. EXPERIMENTAL

4.1. Materials and Methods

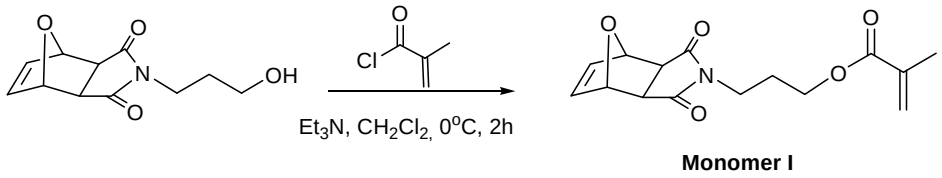
All chemicals were used as received from manufacturer (Merck, Aldrich, Alfa Aesar, Riedel de Haen). Dry solvents (CH_2Cl_2, THF, toluene) was obtained from ScimatCo Purification System, other dry solvents were dried over molecular sieves. Column chromatography was performed using silicagel-60 (43-60 nm). Thin layer chromatography was performed using silica gel plates (Kiesel gel 60 F254, 0.2mm, Merck). Plates were viewed under 254nm UV lamp otherwise plates are developed either by $KMnO_4$ stain. Infrared spectroscopy was carried out on Thermo Scientific Nicolet 380 FT-IR spectrophotometer. Removing water from hydrogels was accomplished with LabConco lyophilizer. ^1H NMR (operating at 400 MHz) were recorded on Varian Mercury-MX in either D_2O or $CDCl_3$ as solvent at the Advanced Technologies Research and Development Center at Boğaziçi University. Thermogravimetric analysis (TGA) were done on TA instrument. The molecular weights were estimated by gel permeation chromatography (GPC) with polystyrene as a standard and with refractive index detector, and the sample was eluted with THF. Elemental analysis data were obtained from Thermo Electron S.p.A. FlashEA® 1112 Elemental Analyzer (CHNS separation column, PTFE; 2 m; 6x5 mm). The dry and wet surfaces of the Hydrogels were observed with an ESEM-FEG/EDAX Philips XL-30.

4.2. Water Soluble Thiol Reactive Polymeric Supports

4.2.1. Synthesis of Latent Reactive Monomer

The furan protected maleimide monomer was prepared as reported before. To a solution of the alcohol (2.00 g, 8.86 mmol) and triethylamine (1.05 mL, 10.63 mmol) in dichloromethane (120 mL) at 0°C, was added methacryloyl chloride (0.91 mL, 9.39 mmol) in

0.1 mL portions over 30 min. The clear solution was stirred for 2 h at 0°C. To the reaction mixture was added dichloromethane (40 mL) and the mixture was washed with saturated $NaHCO_3$ (2 × 40 mL) and H_2O (2 × 40 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated to give a yellow residue that was purified by flash chromatography on SiO_2 (EtOAc:CH_2Cl_2 1:1) affording 2.50 g (96 % yield) monomer as a white waxy solid. Purity has been determined by HPLC (98.4%). ^1H NMR ($CDCl_3$) 6.49 (s, 2H, CH=CH), 6.11 (s,1H, CH_2=C), 5.55 (m, 1H, CH_2=C), 5.24 (s, 2H, CH bridgehead protons), 4.09 (t, 2H, J= 6.2 Hz, OCH_2) 3.59 (t, 2H, J = 7.0 Hz, NCH_2), 2.82 (s, 2H, CH-CH, bridge protons), 1.98–1.91 (m, 5H,$CH_2CH_2CH_2$ and CH_3); ^{13}C NMR ($CDCl_3$) 176.0, 167.1, 136.4, 136.1, 125.4, 80.8, 61.4, 47.3, 35.7,26.6, 18.2; IR (KBr): υ = 1705.8 cm^{-1}.



Monomer I

Figure 4.1. Latent reactive monomer synthesis

4.2.2. Synthesis of TEG Initiator

TEGME (1.60 mL, 10.00 mmol) was dissolved in dry CH_2Cl_2 (100 mL). To the solution of TEGME, Et_3N (1.56 mL, 11.00 mmol) was added and the solution was cooled to 0°C. 2-bromo, 2-methyl propionyl bromide (1.26mL, 10.00 mmol) was added dropwise. The clear solution was stirred for 12 h at 0°C. The mixture was washed with saturated $NaHCO_3$ (2 ×40 mL) and H_2O (2×40 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated in vacuum. The crude product was purified by flash chromatography on SiO_2 (EtOAc : Hexane 3:7) affording 2.02 g of TEG initiator as a yellow liquid. ^1H NMR ($CDCl_3$) :

1.9 (s, (CH3)2–C–Br)), 3.4 (s,CH3O), 3.5 (t, CH2), 3.6 (b, CH2), 3.7 (t, CH2), 4.3 (t, CH2–O–C O).

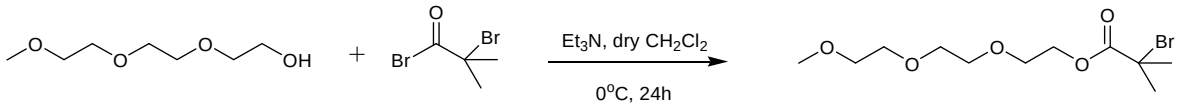


Figure 4.2. Synthesis TEG ATRP initiator

4.2.3. Synthesis of Polymer

In a typical experiment, furan protected maleimide monomer (185.0 mg, 0.63 mmol), 2,2'bipyridine (10 mg, 0.065 mmol), CuBr (4.5 mg, 0.032 mmol) are weighed into a round bottom flask and the flask is connected to Schlenk line. Degassed PEGMEMA (0.72 mL, 2.52 mmol), degassed MeOH (3 ml) and H_2O (3 mL) is added. To the stirring mixture was added TEG initiator (7.90 µl, 0.032 mmol) and stirred at room temperature. The polymerization was monitoring by GPC periodically. After polymerization the reaction mixture is precipitated in diethyl ether and passed through aliminium oxide to remove cupper catalyst. : ^{1}H NMR ($CDCl_3$, ppm) 6.51 (s, 2H, CH=CH), 5.25 (s, 2H, -CH bridgehead protons), 4.04(s, -OCH_2 of PEGMEMA), 3.93 (br s, 2H, -OCH_2), 3.57 (br s, 5H, -OCH3 and -NCH_2), 3.35 (s,-OCH_3), 2.85 (s, 2H, CH-CH, bridge protons), 1.89–0.82 (m, 7H, -$NCH_2CH_2CH_2O$, -CH_2 and -CH_3 along polymer backbone); IR (KBr): v = 1725.5, 1701.1 cm^{-1} .

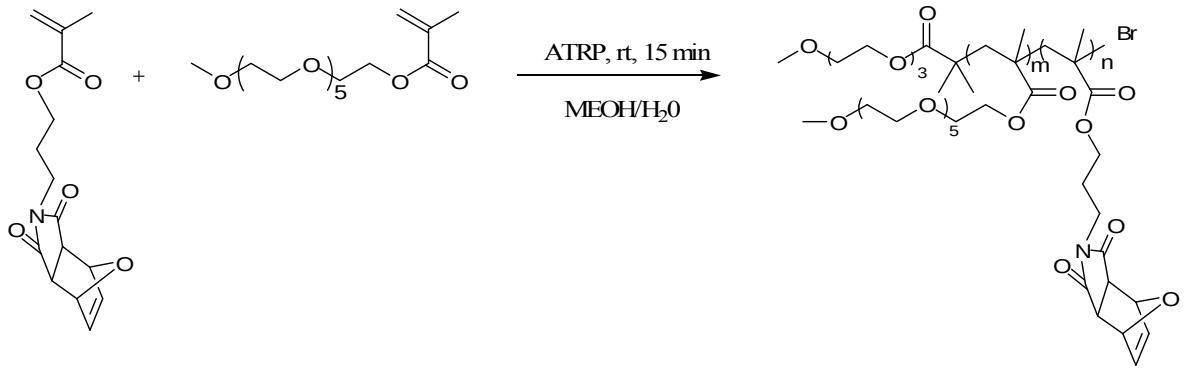


Figure 4.3. Synthesis of protected maleimide containing polymer

4.2.4. Activation of Polymer

Polymer (200.0 mg) was dissolved in dry toluene and heated at 110°C for 4 hours. NMR analysis proved quantitative conversion of the oxabicyclic moiety to the maleimide functional group. NMR for polymer: ^{1}H NMR ($CDCl_3$, ppm) 6.71 (s, 2H, CH=CH, 4.06(s, OCH_2 of PEGMEMA), 3.93 (br s, 2H, OCH_2), 3.57 (br s, 5H, OCH3 and NCH_2), 3.35 (s,OCH_3), 2.85 (s, 2H, CH-CH, bridge protons), 1.92–0.84 (m, 7H, $NCH_2CH_2CH_2O$, CH_2 and CH_3 along polymer backbone); IR (KBr): v = 1707.1 cm^{-1} .

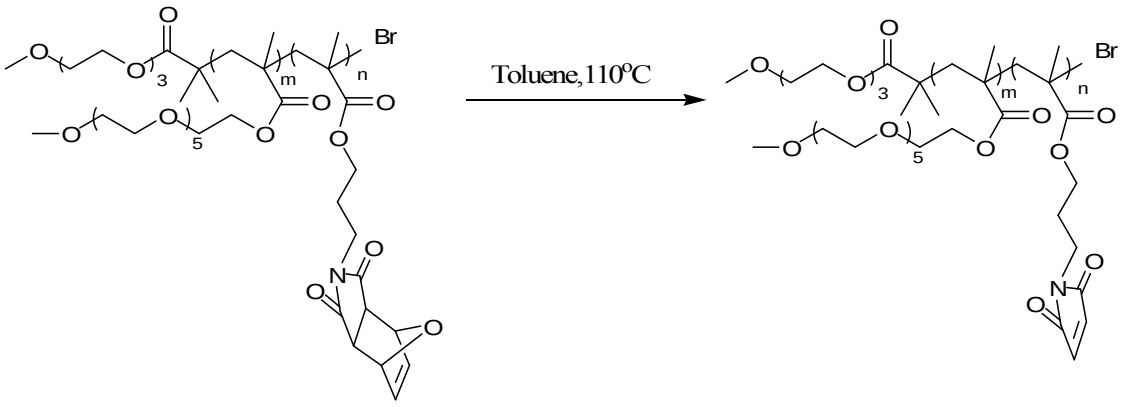


Figure 4.4. Activation of maleimide groups on the side chain of the polymer

4.2.5. Synthesis of PEG Macroinitiator

The PEG macromonomers were prepared as reported before. Briefly, poly (ethylene glycol) monomethyl ether was azeotropically dried using toluene prior to use. PEGME (2.00g, 1.00 mmol) was dissolved in dry THF (30mL). To the solution of PEGME, Et_3N (0.28mL,2.00 mmol) was added and the solution was cooled to 0°C. 2-bromo,2-methyl propionyl bromide (0.86mL,7.00 mmol) was added dropwise. The clear solution was stirred overnight at 0°C. THF is evaporated and dichloromethane (40 mL) was added. The mixture was washed with saturated $NaHCO_3$ (2 ×40 mL) and H_2O (2×40 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated in vacuum. The product is dissolved in minimum amount of dichloromethane and precipitated in diethylether. After vacuum filtration, the product is obtained as a white solid.

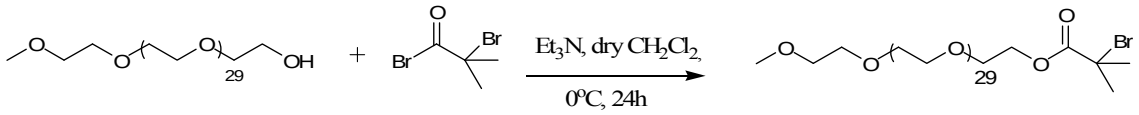


Figure 4.5. Synthesis of TEG initiator for ATRP

4.2.6. Synthesis of Diblock Polymer

In a typical experiment, furan protected maleimide monomer (185.0 mg, 0.63 mmol), 2,2'bipyridine (10 mg, 0.065 mmol), CuBr (4.5 mg, 0.032 mmol) are weighed into a round bottom flask and the flask is connected to Schlenk line. Degassed PEGMEMA (0.72 mL, 2.52 mmol), degassed MeOH (3 mL) and H_2O (3 mL) is added. To the stirring mixture was added PEG macroinitiator (68.8mg, 0.032 mmol) which was dissolved in minimum amount of water and stirred at room temperature. The polymerization was monitoring by GPC periodically.

After polymerization the reaction mixture is precipitated in diethyl ether and passed through aliminium oxide to remove cupper catalyst.

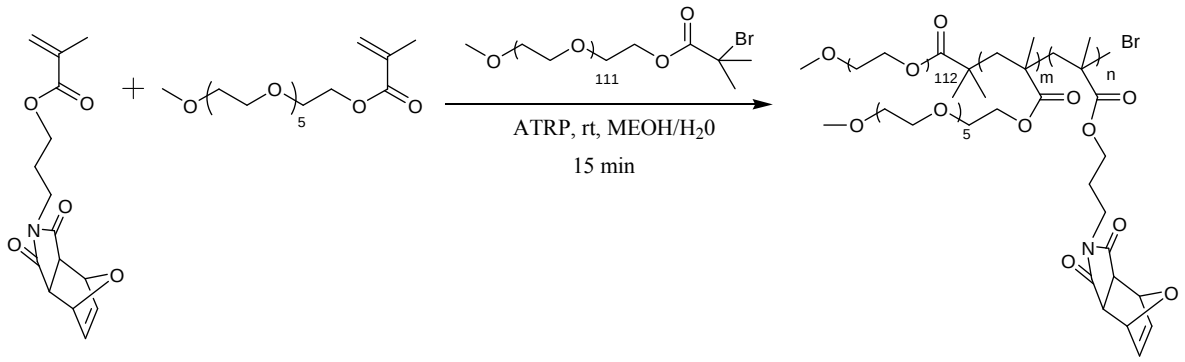


Figure 4.6. Synthesis of diblock polymer

4.2.7. Functionalization with Glutathione

Polymer (10 mg, 0.48μmol) was dissolved in D_2O (0.5 mL) and degassed for 30 minutes. Glutathione was added (0.2 mL from 10 mg/mL stock solution in D_2O) and reacted at room temperature for 24 hours.

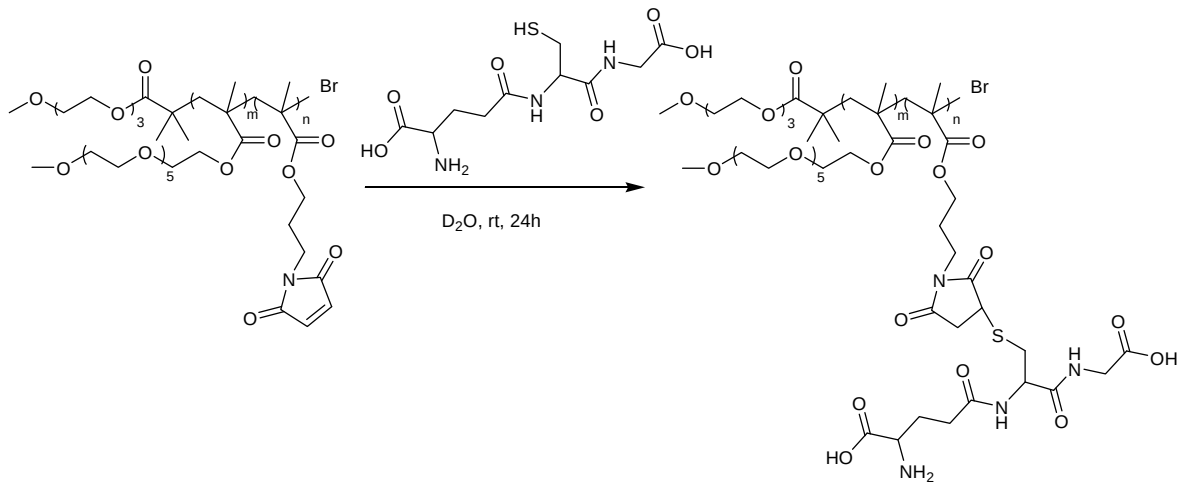


Figure 4.7. Functionalization of the reactive polymer with glutathione

4.3. Thiol Reactive Hydrogels for Bioimmobilization

4.3.1. Synthesis of Hydrogels

Three series of hydrogels are synthesized at both 75 °C and 90 °C containing different monomer ratios as represented in Table 1. Briefly, to a vial containing furan protected maleimide monomer (12.3 mg, 0.042 mmol) and 2,2-azobisisobutyronitrile (AIBN, 4.6 mg, 0.028 mmol) was added degassed PEGMEMA (0.12 mL, 0.42 mmol) and dry, degassed THF (0,14 mL). Then the reaction vials are sealed and placed in oil bath for 30 minutes. The hydrogels were purified by washing several times with methanol under sonication to remove any unreacted monomers. Conversions determined gravimetrically based on gel content were usually above 90%.

4.3.2. Activation of the Hydrogels

Dried hydrogels were heated at 110 °C in anhydrous toluene for 4 hr. Thermogravimetric analysis (TGA) demonstrated that quantitative conversion of the oxabicyclic moiety to the maleimide functional group was achieved. No significant weight loss corresponding to the expected loss of furan was observed upon heating.

4.3.3. Synthesis of Hydrogenated Monomer

4.3.3.1. Hydrogenation of the Cyclo Adduct: To a solution of the alcohol (0.5 g, 2.24 mmol) was added Pd on C (0.05g, 10% by weight). The mixture was dissolved in a mixture of ethanol:ethyl acetate (3 mL : 7 mL) and purged with H_2 using H_2 baloons. The mixture was reacted overnight. After the reaction the mixture was filtered to remove Pd on C and concentrated in vacuum to obtained hydrogenated alcohol.

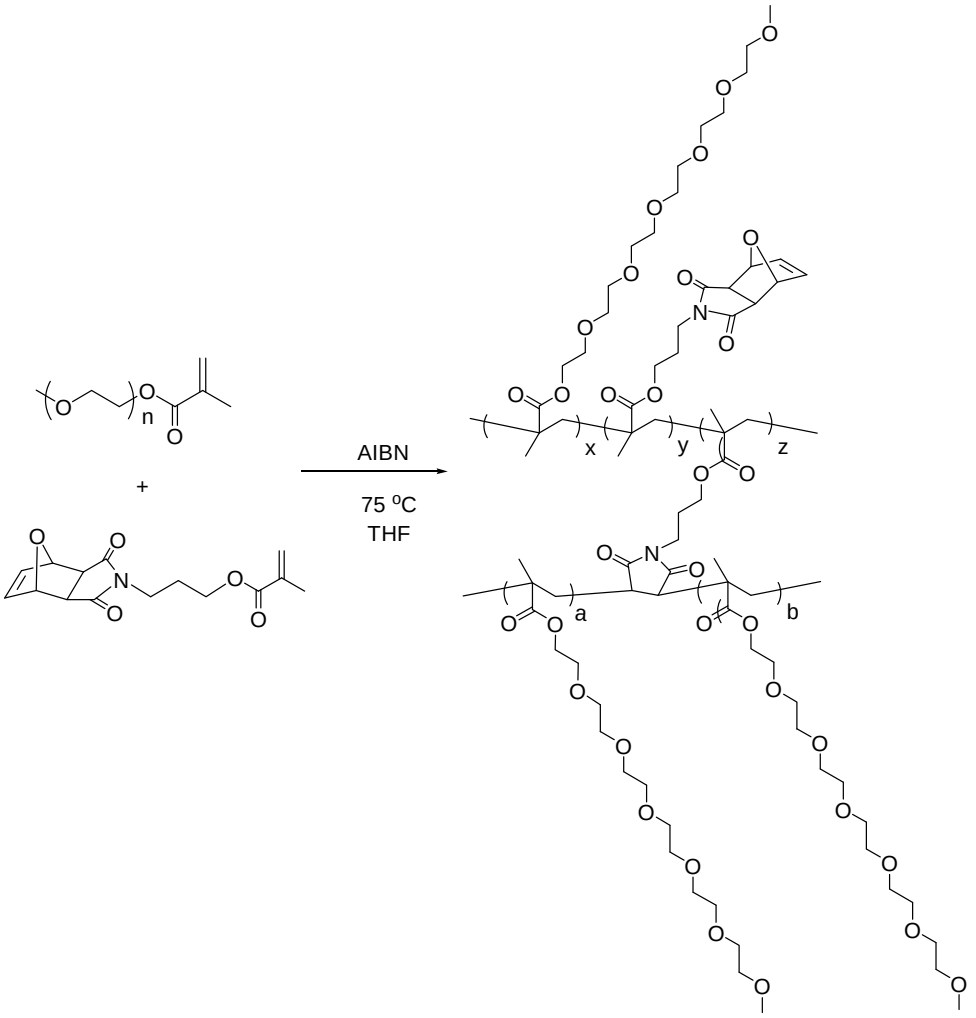


Figure 4.8. Representation of Hydrogel Synthesis

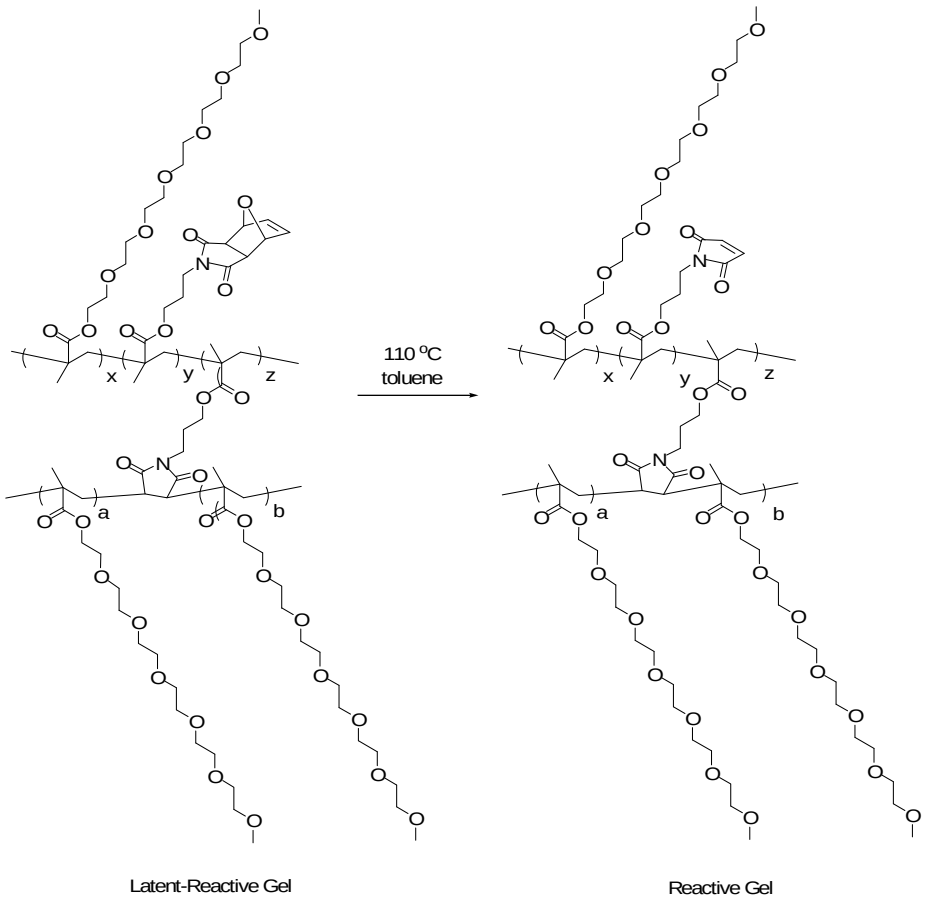


Figure 4.9. Activation of hydrogels

4.3.3.2. Synthesis of the Hydrogenated Monomer: To a solution of the hydrogenated alcohol (0.20 g, 0.89 mmol) and triethylamine (0.15 mL, 1.08 mmol) in dichloromethane (60 mL) at 0°C, was added methacryloyl chloride (0.09 mL, 0.93 mmol) over 30 min. The clear solution was stirred for 2 h at 0°C. To the reaction mixture was added dichloromethane (40 mL) and the mixture was washed with saturated $NaHCO_3$ (2 ×40 mL) and H_2O (2×40 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated to give a yellow residue that was purified by flash chromatography on SiO_2 (EtOAc:CH_2Cl_2, 1:1) affording 2.50 g (96 % yield) monomer as a white waxy solid. ^1H NMR ($CDCl_3$), 6.11 (s,1H, CH_2=C),

5.55 (m, 1H, CH_2=C), 4.84 (s, 2H, -CH bridgehead protons), 4.08 (t, 2H, J= 5.9 Hz,-OCH_2) 3.56 (t, 2H, J = 6.4 Hz, -NCH_2), 2.84 (s, 2H, CH-CH, bridge protons), 1.93–1.57 (m, 5H, CH_2-**CH_2**-CH_2 , -CH_3, CH_2-CH_2).

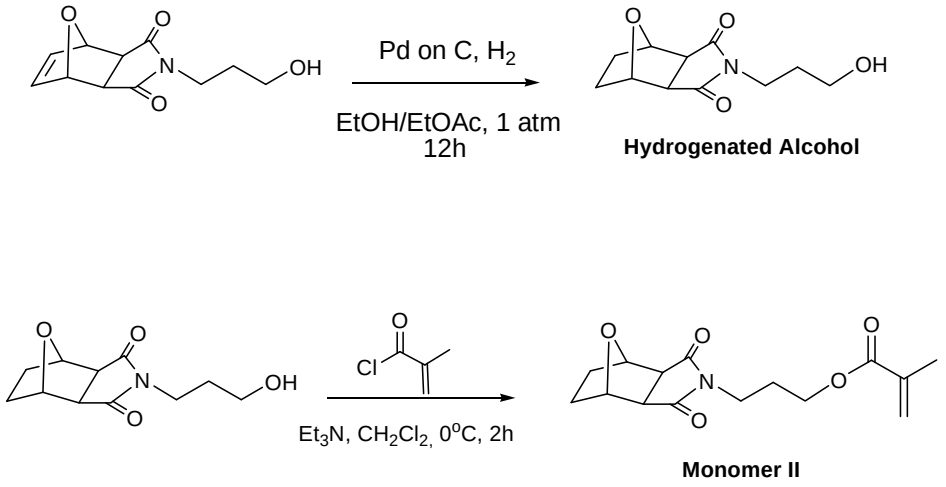


Figure 4.10. Synthesis of hydrogenated monomer

4.3.4. Swelling Studies

A circular piece of purified and dried hydrogel was transferred to a flask containing distilled/deionized water at room temperature. The mass of the hydrogel sample is recorded regularly after removing the hydrogel from solution and drying the surface with a filter paper.

4.3.5. Scanning Electron Microscopy

Swollen hydrogels are lyophilized and scanning electron microscopy is used to characterize the morphology of the hydrogels. The hydrogel is immersed in liquid nitrogen

and broken and images are taken using ESEM-FEG/EDAX Philips XL-30 (Philips, Eindhoven, The Netherlands) instrument using an accelerating voltage of 10 kV.

4.3.6. Functionalization with Fluorescent Dye

BodipyC10SH.Hydrogels are functionalized with a fluorescent dye, BodipyC10SH after activation. To a degassed solution of BodipyC10SH (2.73 mg) in dry THF (0,5 mL) was added a dried sample of H1 (10 mg, 2.21 % furan) and reacted overnight. Hydrogel was washed several times with THF and fluorescent images were taken. As a control the same hydrogel was incubated with a solution of BodipyC10Br and after several washing.

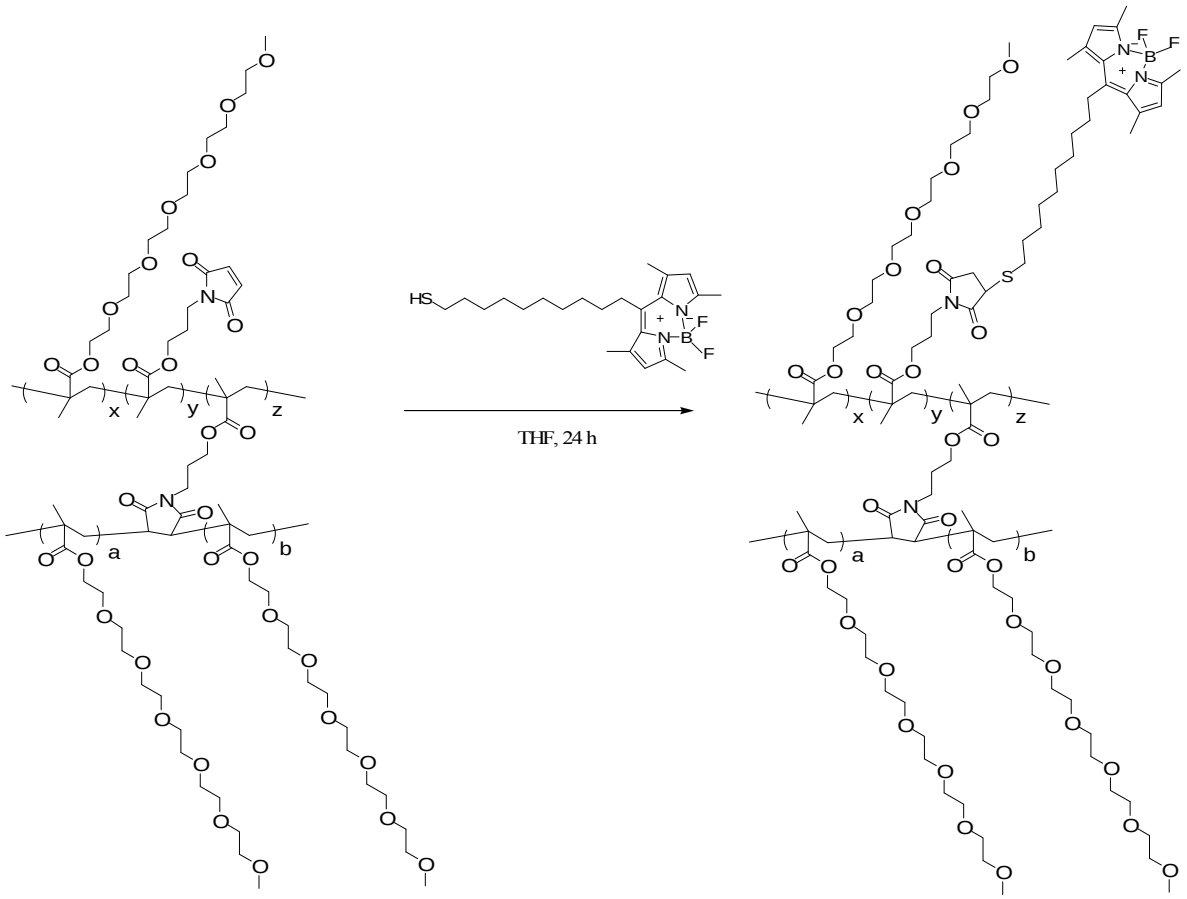


Figure 4.11. Conjugation with a fluorescent dye

4.3.7. Functionalization with an Enzyme, Streptavidin

To a degassed solution of BiotinSH (4.20 mg) in MeOH (0,5 mL) was added a dried sample of H1 (10 mg, 2.21 % furan) and reacted overnight. Hydrogel was washed several times with MeOH and incubated with a solution of FITC conjugated Streptavidin (0,1 mg/mL PBS) for 20 minutes. After incubation sample was washed with PBS and deionized water several times and fluorescent images were taken. As a control a hydrogel which was not biotinylated was incubated with a solution of Streptavidin and after washing steps fluorescent images were taken.

5. CONCLUSIONS

Novel thiol reactive polymers and hydrogels have been synthesized incorporating a maleimide group in the side chain of the polymer along with poly (ethylene glycol) .

In the first part of the study, water soluble thiol reactive polymers are synthesized via atom transfer radical polymerization of furan protective maleimide polymer and poly (ethylene glycol) methacrylate. The maleimide groups were obtained quantitatively in their native form by heating the polymer after polymerization. To show that this novel polymer could be functionalized with desired functional groups using a thiol based nucleophile, a thiol containing compound, glutathione was reacted with maleimide polymer. The maleimide groups in the polymers were derivatized with thiol moieties efficiently at room temperature without need of any extra reagents. As an extension of this work diblock copolymers bearing maleimide groups in the side chain was synthesized utilizing poly (ethylene glycol) macroinitiators.

In the second part, hydrogels containing maleimide functional groups are synthesized using a novel Diels-Alder/retro Diels-Alder strategy. The hydrogels obtained were efficiently functionalized with small molecules such fluorescent thiol containing dye molecule BODIPY and thiol containing biotin ligand. The biotinylated hydrogel was used to immobilize the enzyme streptavidin, which can be used for further functionalization of the hydrogel. Excellent antibiofouling property of PEG based inert framework provides negligible unspecific adsorption of enzyme under study.

APPENDIX

^{1}H NMR and IR data of the newly synthesized compounds are included.

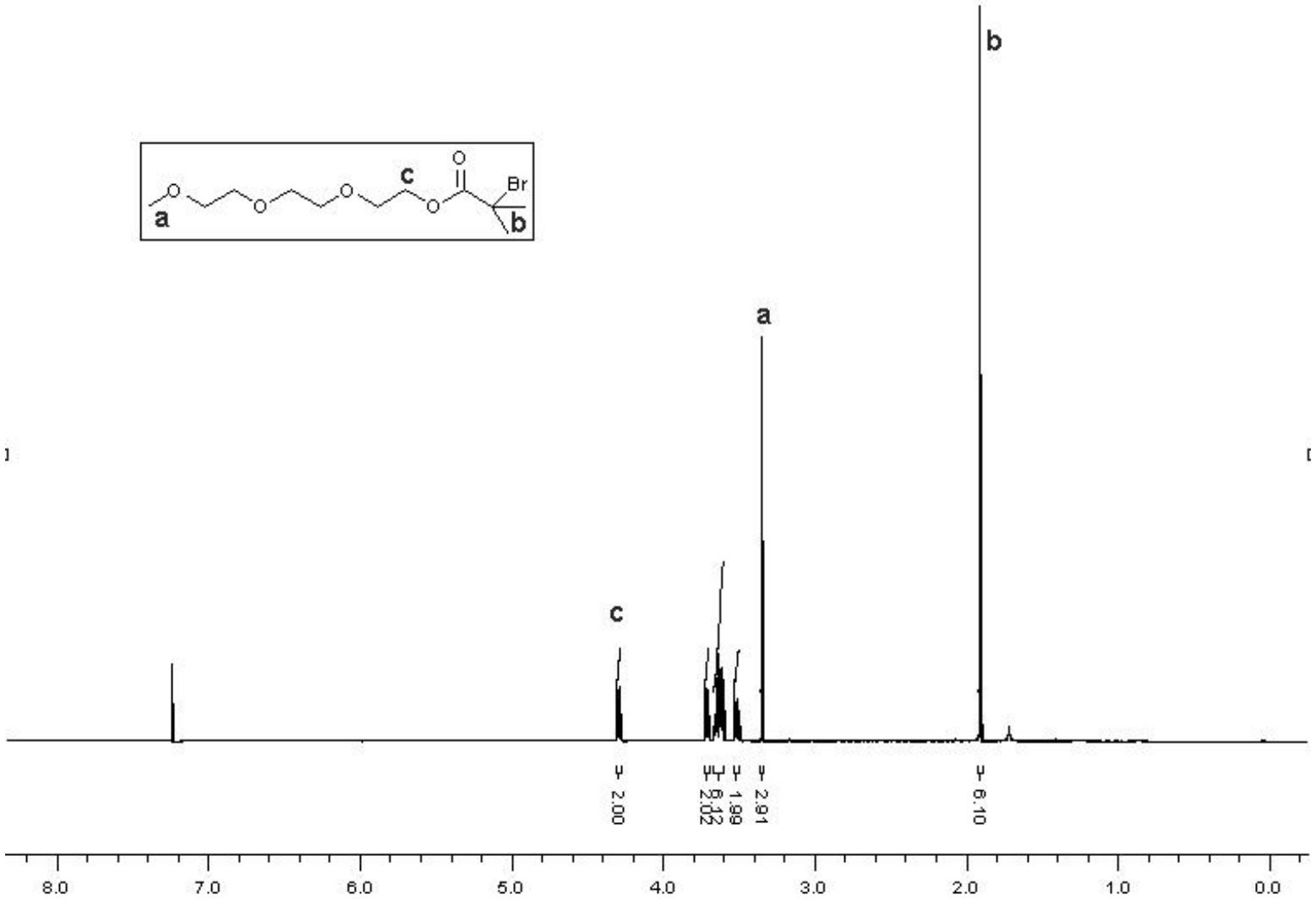


Figure A.1. ^{1}H NMR spectrum of TEG initiator

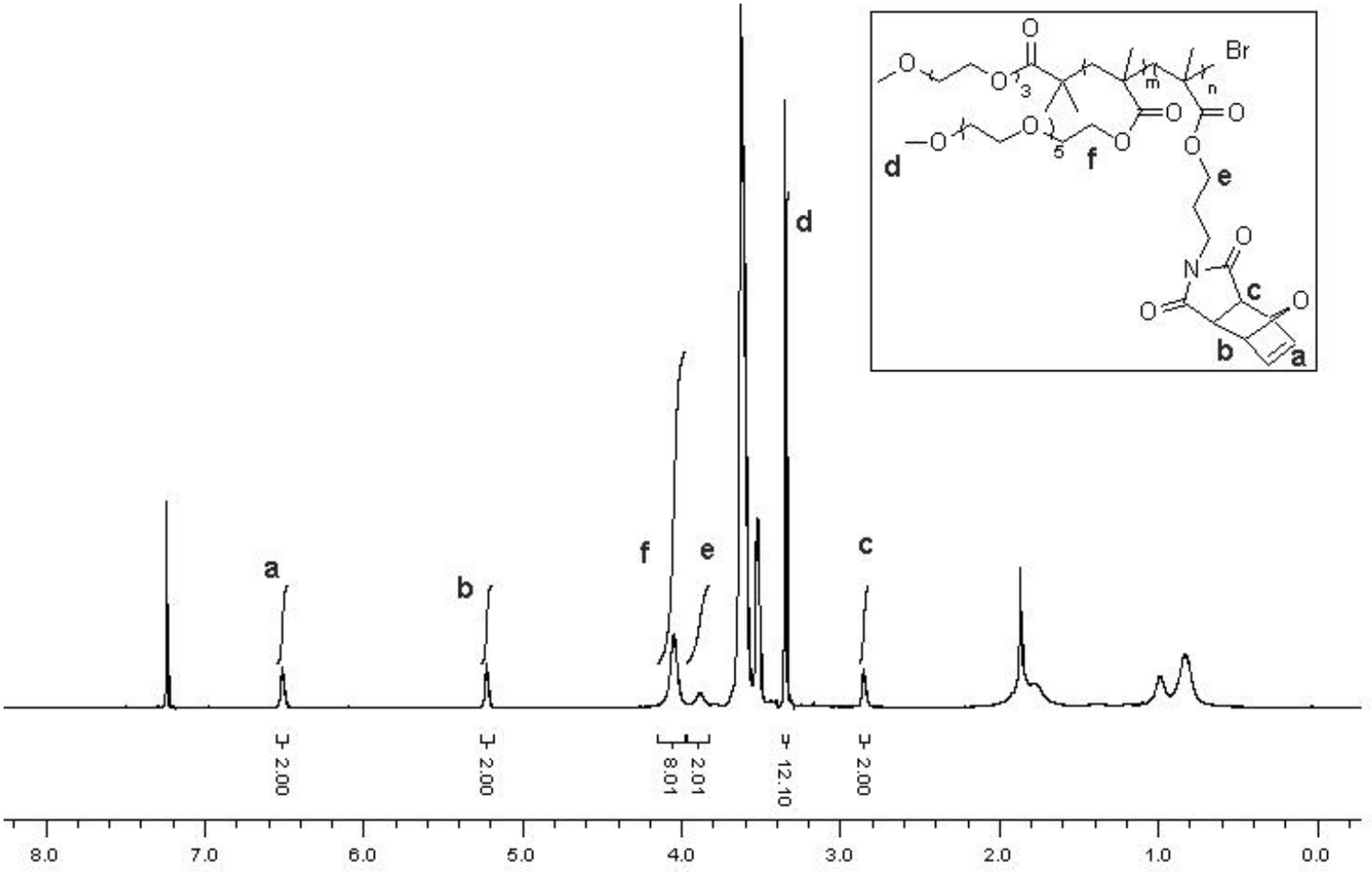


Figure A.2. ^{1}H NMR of spectrum **P3**

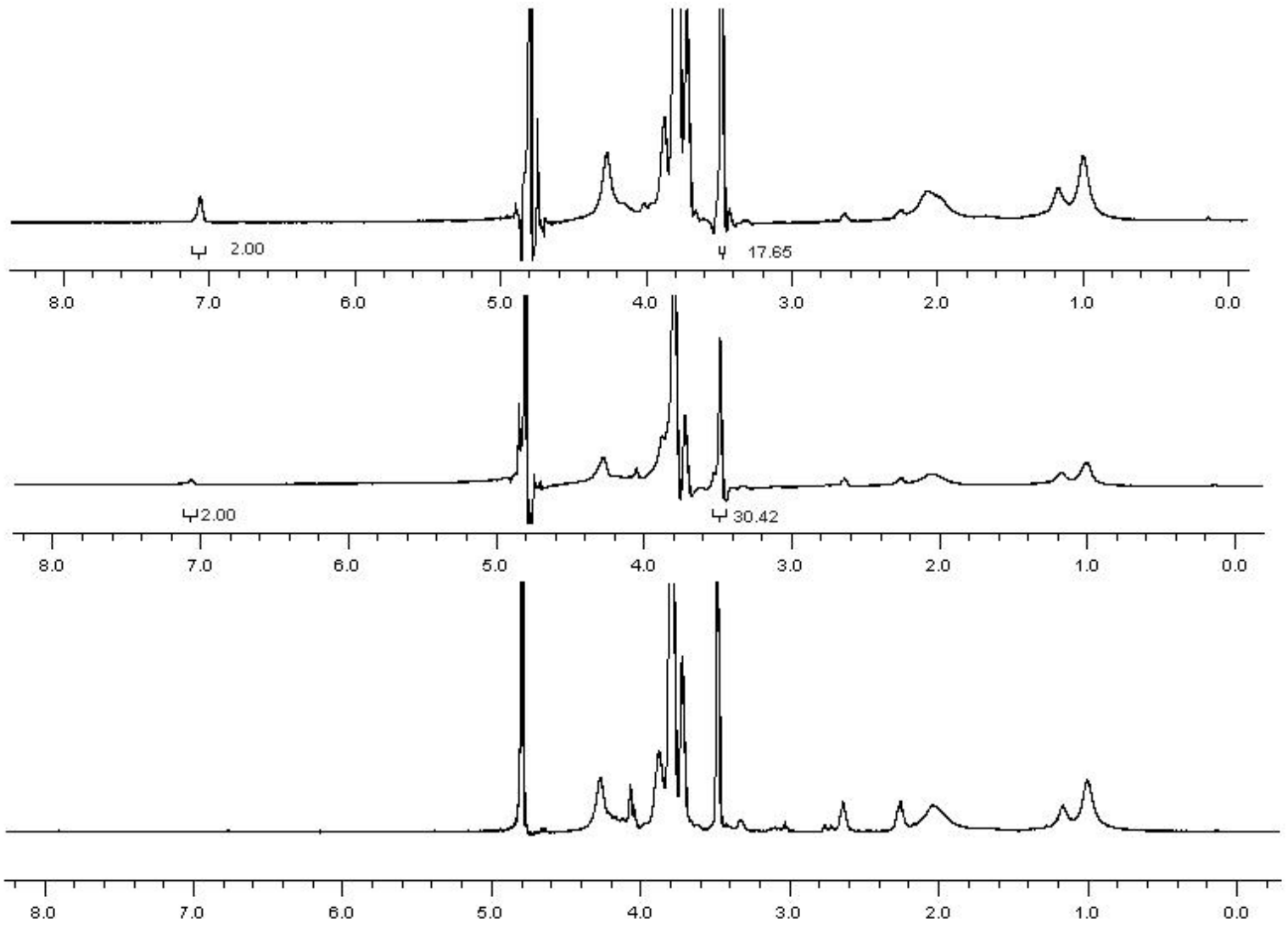


Figure A.3. ^{1}H NMR spectrum of **P3** conjugated to 0.3, 0.6, 1 eq of glutathione respectively

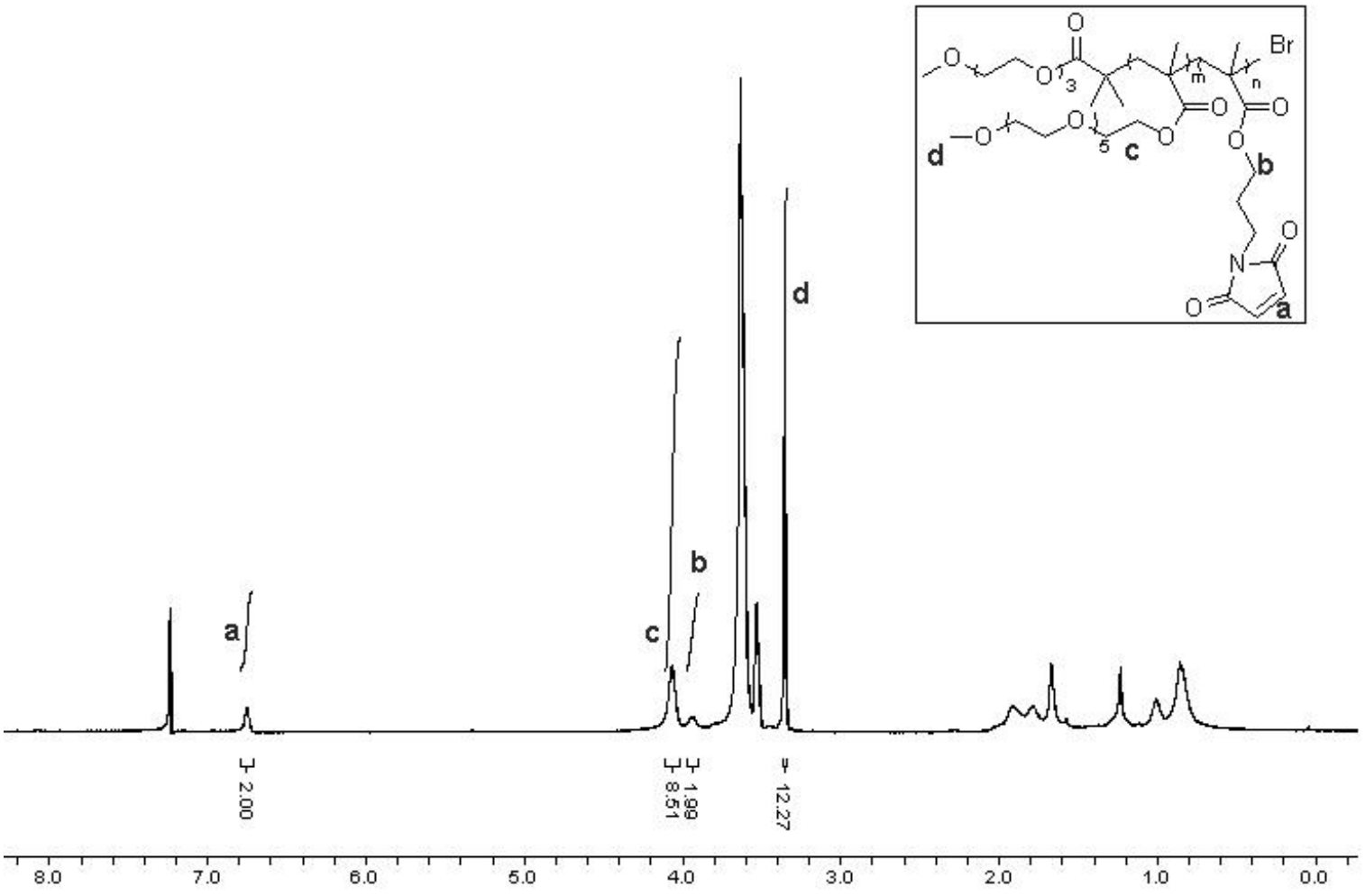


Figure A.4. ^{1}H NMR spectrum of **P5**

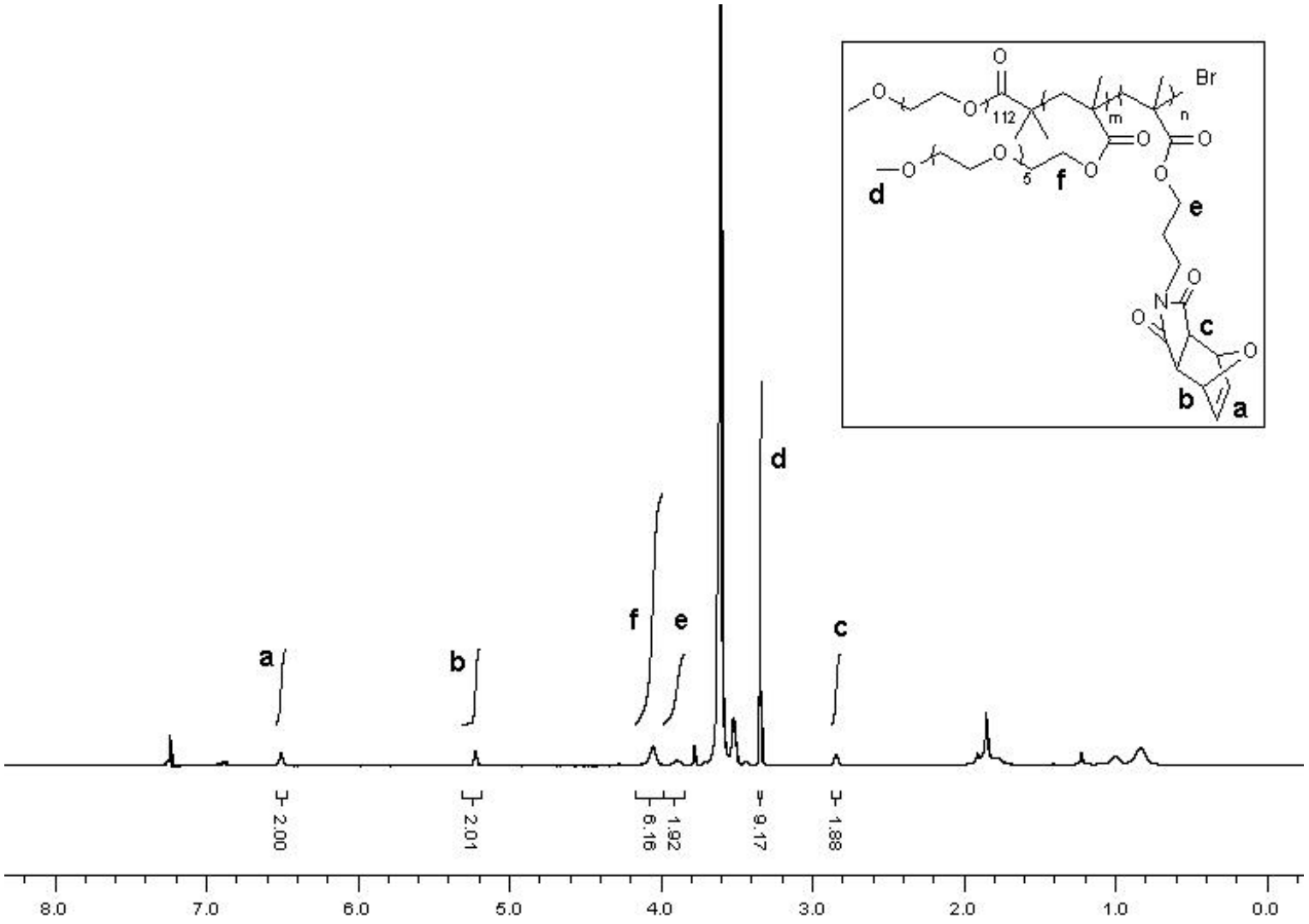


Figure A.5. ^{1}H NMR spectrum of **P 12**

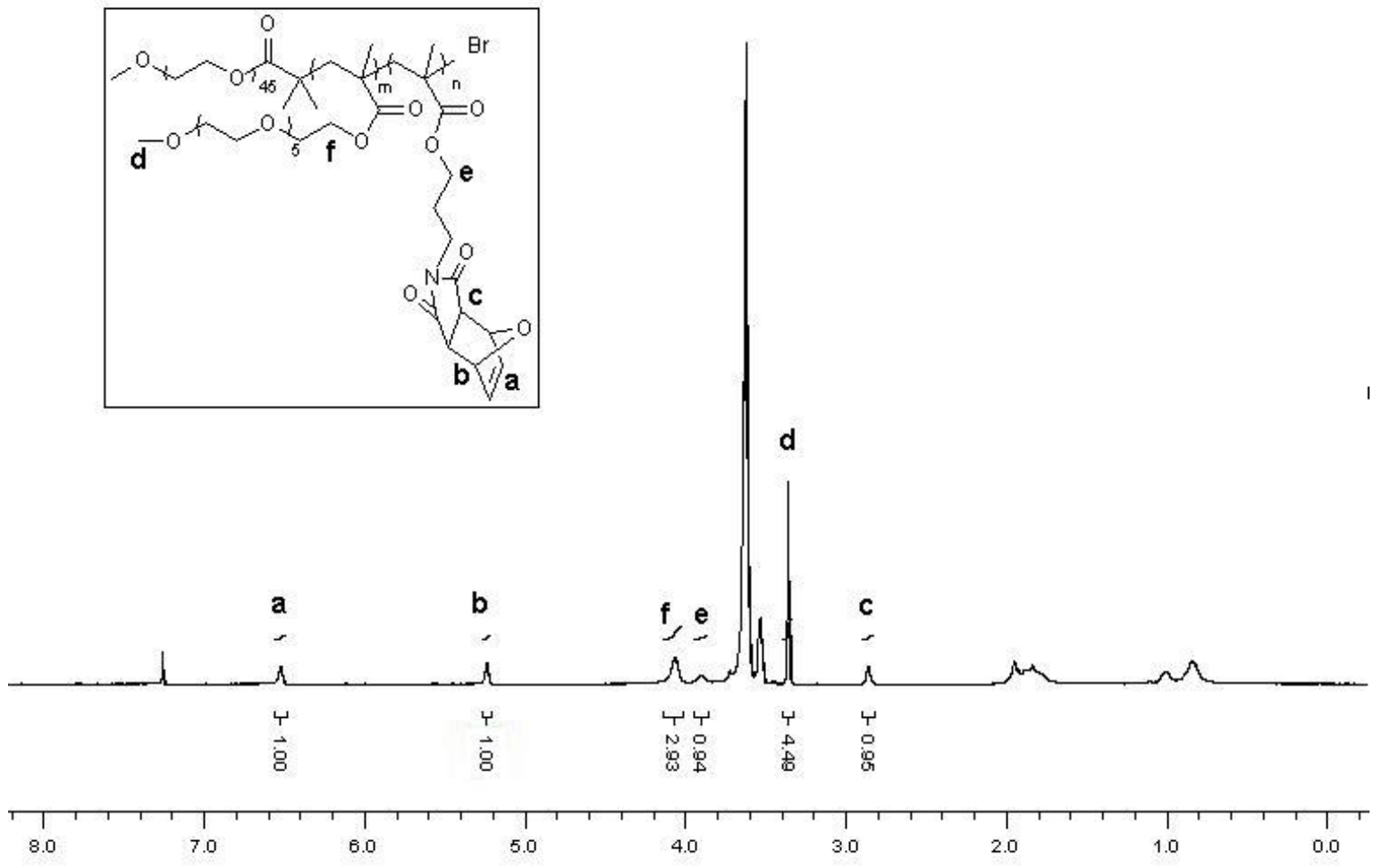


Figure A.6. ^{1}H NMR of **P 14**

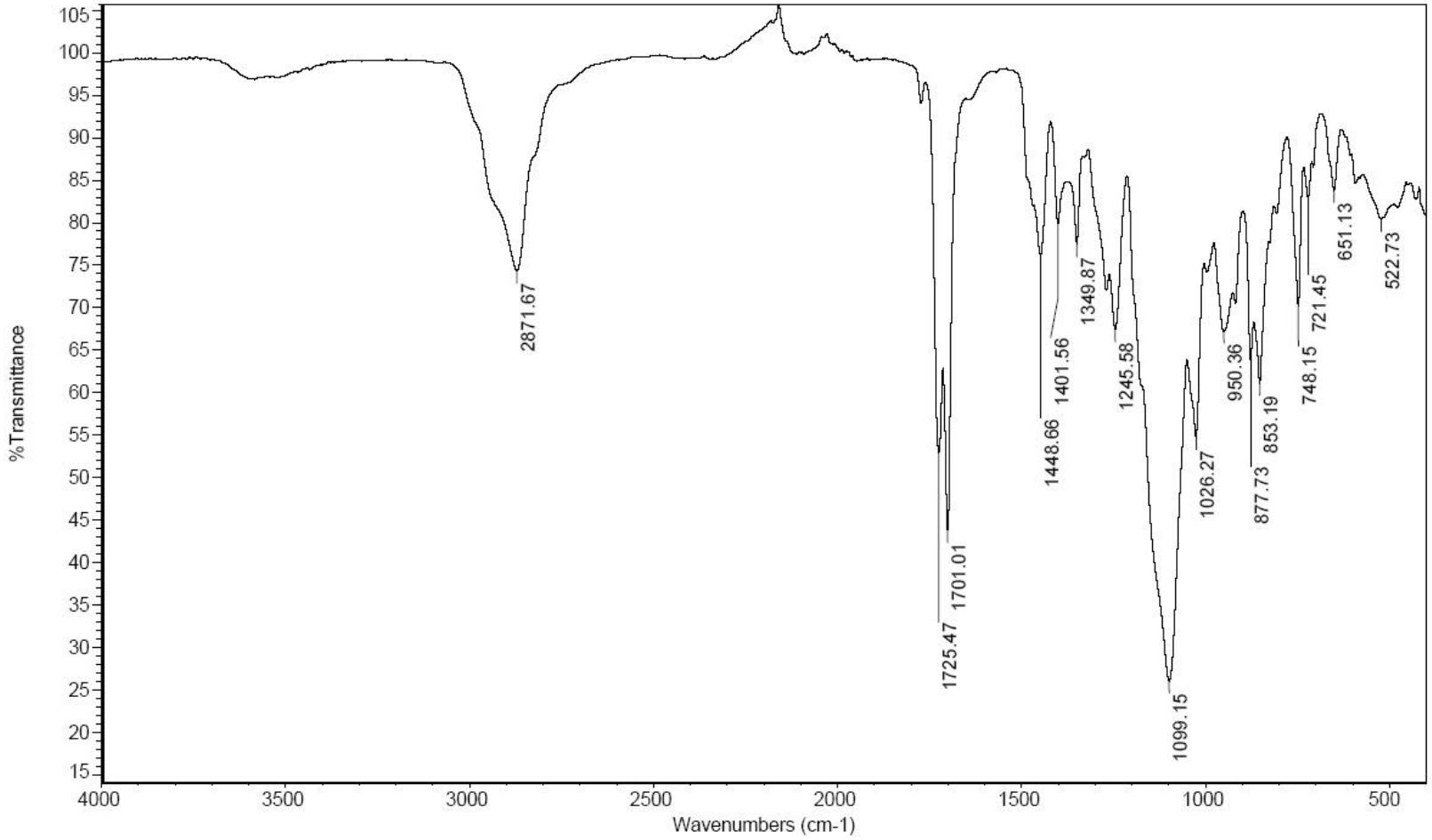


Figure A.7. IR spectrum of **P3**

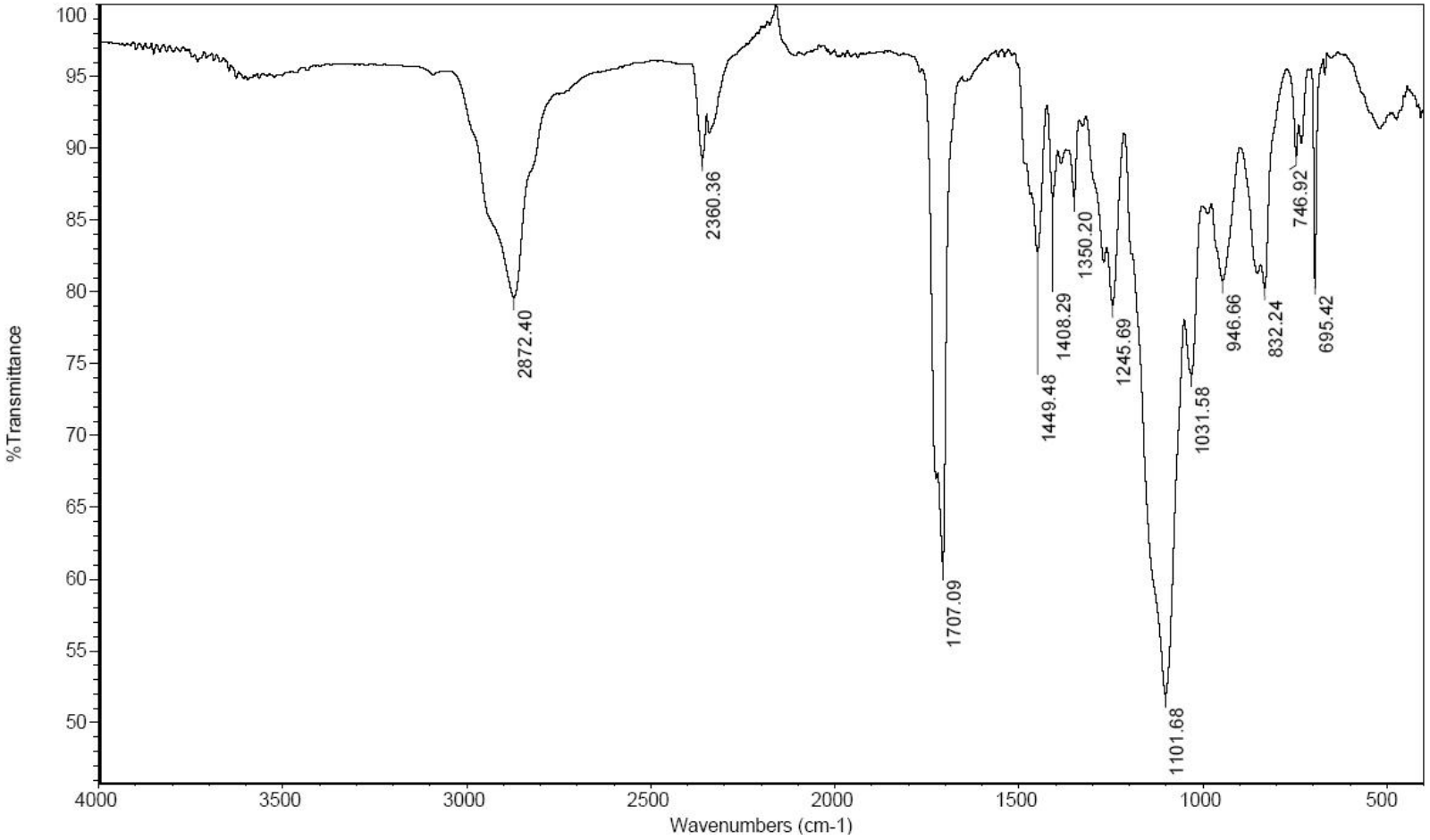


Figure A.8. IR spectrum of **P5**

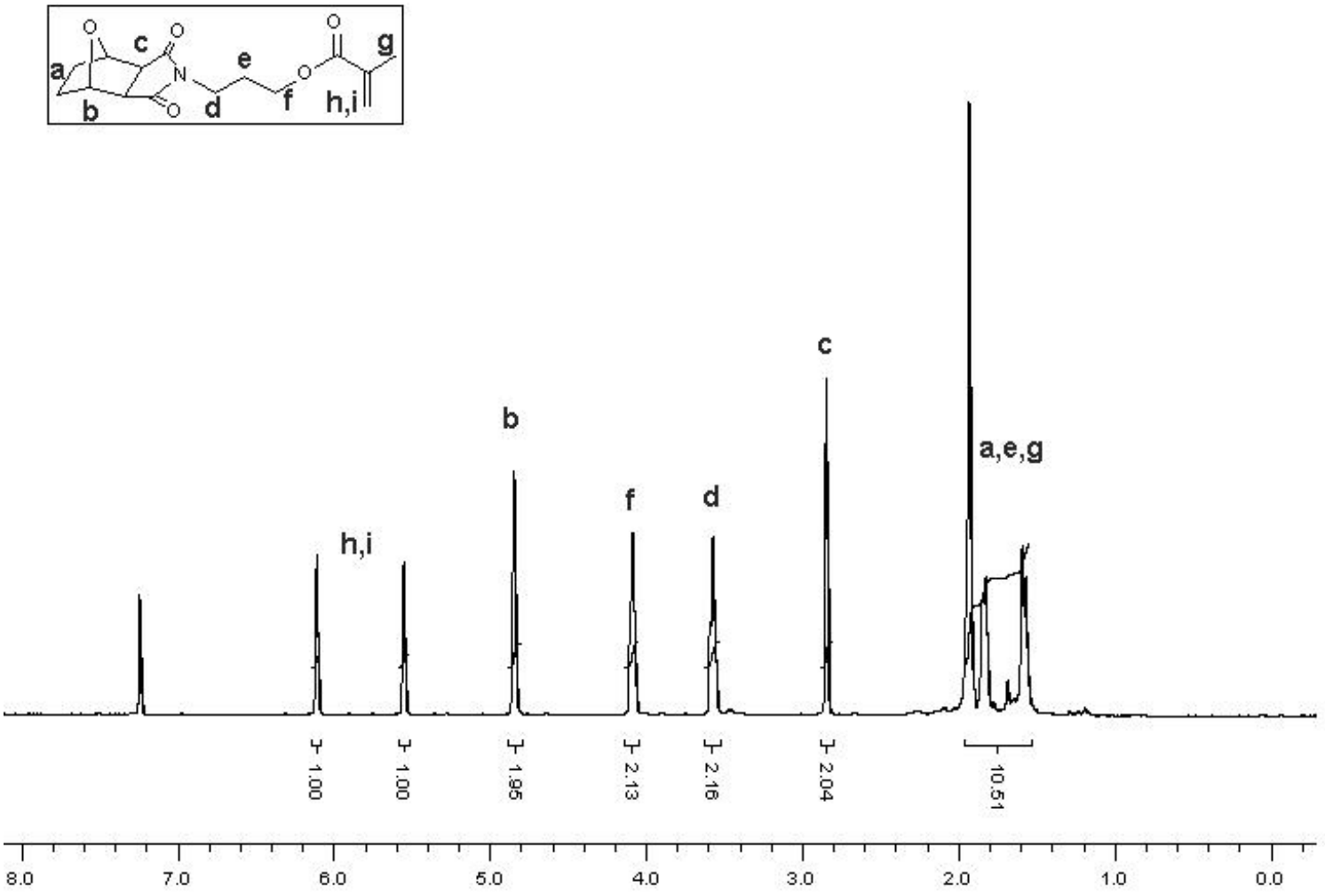


Figure A.9. ^{1}H NMR spectrum of Hydrogenated Monomer

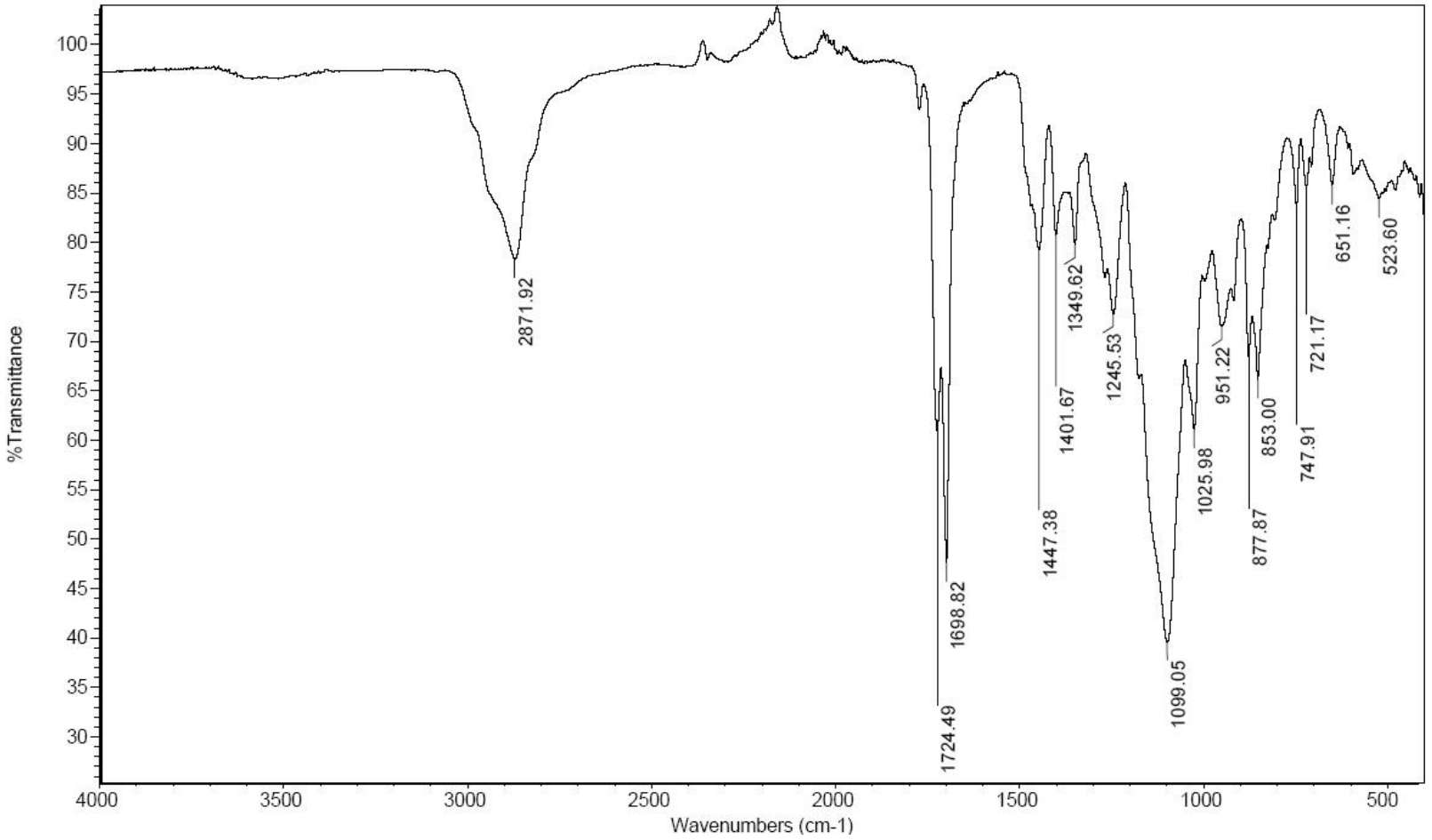


Figure A.10. IR Spectrum of **H3**

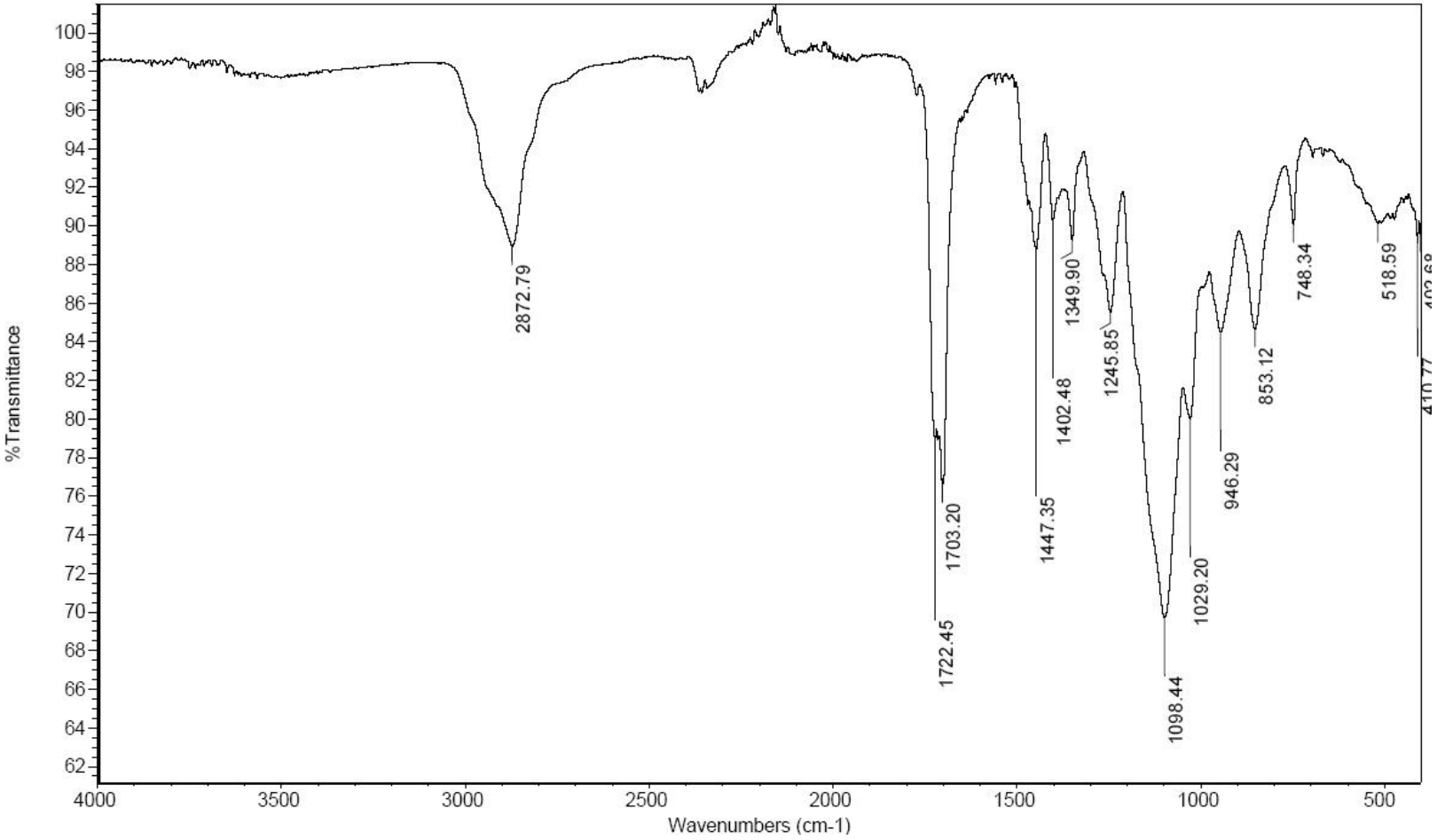


Figure A.11. IR spectrum of retro **H3**

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