PROTEIN RELEASE FROM REDOX RESPONSIVE HYDROGELS

by

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TO MY FAMILY AND MY GIRLFRIEND

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ABSTRACT

Hydrogel networks that can enable slow release of therapeutic proteins are attractive candidates for various biomedical applications. For example, these materials can serve as valuable coatings on medical implants that can present proteins to the cellular environment to enable adaptation of the foreign implant materials in the body. These materials can also be used as post-operative fillers to release therapeutic peptides and proteins to eradicate any residual tumorigenic cells. In this study, three dimensional hydrogel networks containing degradable disulfide bonds have been prepared using the Diels-Alder click chemistry. Hydrogels containing different amount of degradable crosslinker were synthesized via the Diels-Alder reaction between furan bearing PEG-based copolymer and disulfide containing bismaleimide crosslinker. Furan bearing copolymer was synthesized via single electron transfer-living radical polymerization (SET-LRP) technique using poly(ethylene glycol) methyl ether methacrylate (PEGMEMA) and furfuryl methacrylate (FuMA). Rheological analysis of different hydrogels with various crosslink densities was carried out during their formation and then their degradation rates were examined in PBS solution and reducing agent containing solution. FITC-labeled bovine serum albumin was loaded into the hydrogels physically during their fabrication. Protein release studies for hydrogels with different crosslink density were carried out in the presence and absence of reducing agent to demonstrate that the extent of release was tunable.

ÖZET

Tedavisel proteinlerin yavaş salınımını yapabilen hidrojeller bir çok farklı aplikasyon için iyi birer adaydırlar. Bu malzemeler, örneğin, vucudun yabancı madde olarak gördüğü implant materyallerin hücresel ortama adapte olabilmesi için bu medikal implantları kaplamak koşuluyla kullanılabilirler. Bu malzemeler aynı zaman da operasyon sonrası doldurucu olarak tümör oluşturucu hücre kalıntıları yok etmek için iyileştirici peptit ve protein salınımında kullanılabilir. Bu çalışmada kırılabilir bisülfit bağı içeren üç boyutlu hidrojel ağları Diels-Alder klik kimyası kullanılarak hazırlandı. Farklı oranda çapraz bağlayıcı içeren hidrojeller, furan grubu içeren PEG temelli polimer ve bisülfit içeren bis maleimide çapraz bağlayıcı kullanılarak Diels-Alder tekniğiyle sentezlendi. Furan grubu içeren polimerler SET-LRP tekniği kullanılarak PEGMEMA ve FuMA monomerlerinden sentezlendi. Çeşitli çapraz bağlayıcı oranlarındaki farklı Hidrojellerin reolojik analizleri yapıldı ve hidrojellerin bozulma oranları PBS ve indirgeyici madde içeren solüsyonlarda ayrı ayrı incelendi. Protein salınım çalışmaları için FITC bağlı bovine serum albümin fiziksel olarak hidrojellere yüklendi. Farklı çapraz bağlayıcı oranlarındaki hidrojellerden protein salınımı çalışmaları indirgeyici madde içeren ortamda ve indirgeyici madde içermeyen ortamda protein sanılınımının ayarlanabilir olduğunu göstermek incelendi.

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1. INTRODUCTION

1.1. 3D Networks

Three-dimensional (3D) hydrogel networks play a crucial role in the field of tissue engineering, controlled drug delivery, sensors and implant materials. Large surface area/volume ratio, biocompatibility and the degradability of 3D scaffolds make these networks highly attractive for various applications [1]. Ideal scaffolds for cell and tissue culture based applications should exhibit special structural and biosimilar characteristics for desirable applications [2]. Although natural scaffolds such as fibronectin and collagen are known biosimilar materials, their structural characteristics are not at desirable level. However, it is possible to create scaffolds with varying mechanical strength and biological properties by using synthetic polymeric scaffolds such as hydrogels and fibers [3].

In recent years, hydrogels have attracted a lot of attention as crosslinked 3D networks due to their facile fabrication, scalability and low cost [4]. Hydrogels are polymeric networks, which have hydrophilic groups and domains that causes their hydration in aqueous media, therefore these materials can absorb and store large amount of water. At the same time, from a rheological perspective, high crosslink densities can help obtain stiffer networks which have high mechanical strength but low water uptake efficiency, so characteristics of these materials can be tuned for a wide variety of application [5]. An interaction between polymeric chains has to be present in the hydrogels in order to prevent dissolution of hydrophilic polymer chains in aqueous environment. To date, a variety of interactions such as chemical bonding, electrostatic attraction, hydrogen bonding and hydrophobic interactions have been employed to obtain crosslinked polymers. In chemically crosslinked hydrogels, chemical bonds are present between polymer chains; whereas in physically crosslinked hydrogels, electrostatic or hydrophobic interactions keep the hydrophilic polymer chains together. It is also possible to introduce responsive behavior in hydrogels by modulating the nature of chemical or physical interactions using external stimuli such as light,

temperature or redox change. For example, Harada and coworkers reported a redox-responsive hydrogel system using molecular recognition of beta-cyclodextrin. In their system, due to physical interaction between long alkane chains, dodecyl-modified poly(acrylic acid) is crosslinked and in the presence of beta-cyclodextrin, this crosslinking is removed. Incorporating of electroactive ferrocene units into the medium keeps beta-cyclodextrins occupied and crosslinking reoccurs. They also succeed to regain beta-cyclodextrin by oxidizing the ferrocene-carboxylic acid and the free beta-cyclodextrins react with long alkyl chains and degrade the hydrogel. (Figure 1.1) [6].

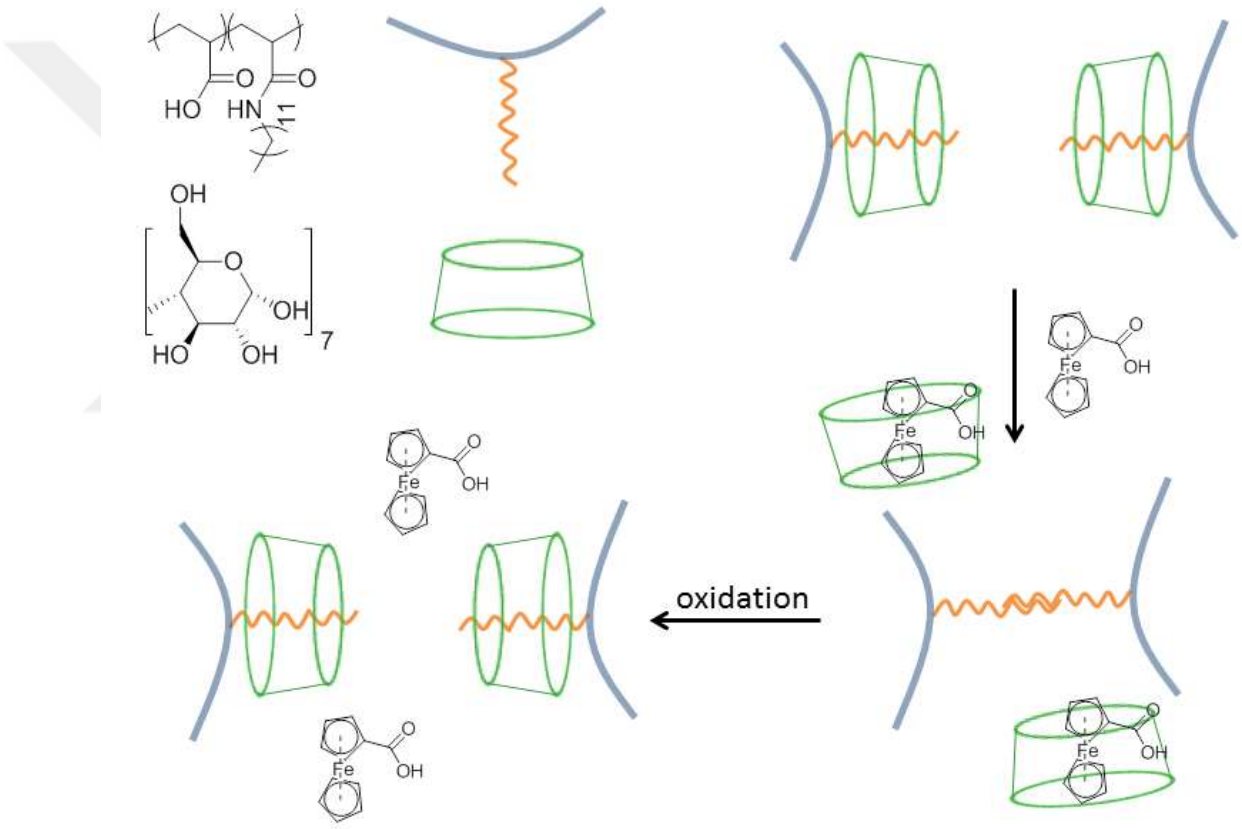

Figure 1.1. Redox-responsive physically crosslinked hydrogel

It must be noted that in general chemically crosslinked hydrogels are more robust than physically crosslinked hydrogels due to their high stability. Chemically crosslinked hydrogels can be obtained by either direct polymerization of monomers in the presence of a crosslinker or through crosslinking of reactive group containing polymers. Peppas and coworkers reported 3D network with controllable swelling and release profile by

crosslinking 2-hydroxyethyl methacrylate and methacrylic acid monomers. In their study, they demonstrated that control over swelling degree of hydrogel allows control over the drug release from network [7]. In general, photopolymerization of water soluble monomers gives non-uniform heterogeneous gels due to uncontrolled crosslinking and these systems ends up with unpredictable mechanical strength.

Using polymers instead of monomers to build hydrogel networks brings some advantages such as adjustable mechanical strength and control over number of functional groups. Polymers that are used in fabrication of hydrogel can be divided into two groups; side chain reactive polymers and end group reactive polymers. Multi-arm polymers with reactive end groups are one of most common materials employed to obtain ‘well-defined’ network. Here, the term “well-defined” implies that the connectivity of junction points of polymers and cross-linkers are precisely known. Recently, Hawker and coworkers reported a well-defined hydrogel network using Huisgen type azide-alkyne cycloaddition based ‘click’ chemistry. In their study, alkyne and azide functionalized poly(ethylene glycol) based polymers were utilized to obtain crosslinked network system with adjustable mechanical strength (Figure 1.2).

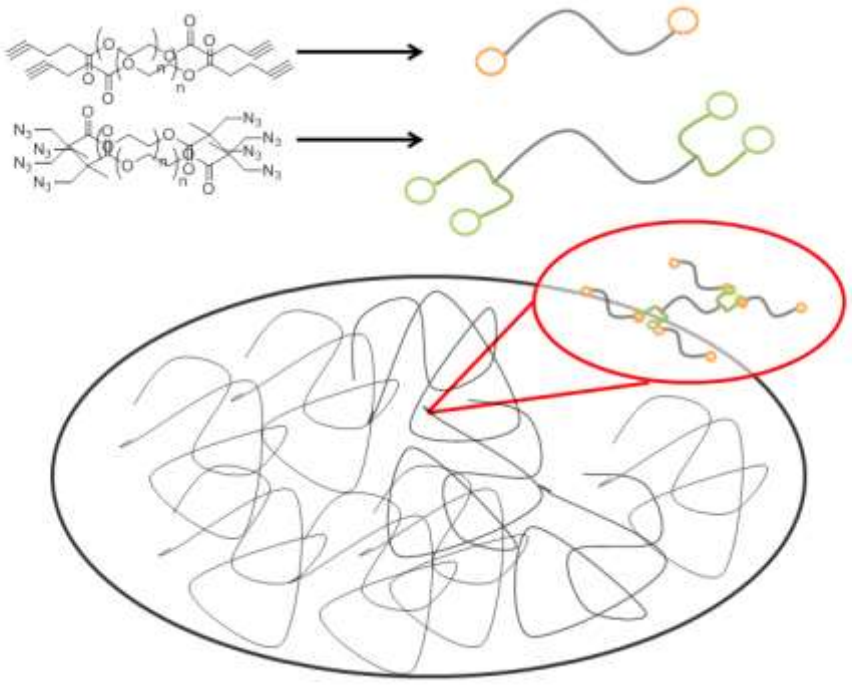

Figure 1.2. Hydrogel synthesis from modified PEG based polymers via click chemistry

Side chain reactive polymers are also widely used building blocks for fabrication of hydrogels. Using reactive functional groups as side-chains brings advantages such as availability of a large number of reactive groups, some of which can be used for crosslinking while the residual ones can be utilized for incorporating various functional molecules into the hydrogel. Furthermore, the number of reactive groups is important for not only adding functionality but also modulate the mechanical strength of hydrogels. Mechanical strength of the hydrogel matrix is directly related to number of junction point in the system. Cui and coworkers reported a nice example of side chain reactive polymer and bifunctional crosslinker based hydrogel system. They used poly(N-isopropylacrylamide)-based furan bearing polymers and bis-maleimide crosslinker to fabricate thermo-responsive hydrogels. When they utilized polymers with higher furan/NIPAAM molar ratio, swelling degree of hydrogels decreased due to the higher number of crosslinking points [8].

1.2. Click Chemistry

'Click' chemistry, a concept introduced by Sharpless about a decade ago has led to an increased impact of organic chemistry on polymer chemistry. 'Click' chemistry embodies reactions that proceed with high yield and selectivity under mild reaction conditions [9]. Design and synthesis of complex macromolecules and polymers are significantly challenging due to steric issues. High efficiency of 'click' conjugations combined with the lack of side products and easy purification had led to widespread use of click chemistry in polymer synthesis. Selectivity is very important in synthesis and modification of macromolecules because it would be extremely challenging to purify macromolecules in the presence of side products. Therefore, judicious choice of suitable 'click' strategy is essential for obtaining polymeric materials with high efficiency.

As mentioned earlier, one of the common approaches in fabrication of chemically crosslinked hydrogels is using 'click' chemistry to crosslink polymers containing appropriate reactive functional groups. One of most popular 'click' reaction that has been used in crosslinking hydrophilic polymer chains is the copper(I)-catalyzed regiospecific formation of 1,2,3-triazoles from azides and terminal acetylenes. Metal

catalyst requirement for the reaction makes it less attractive in some cases. Other common ‘click’ chemistry reaction is the thiol-ene photo-click chemistry which proceeds rapidly under UV irradiation. Michael addition strategies are also popular due to their high reaction velocity even without catalyst or activator. Diels-Alder (DA) ‘click’ chemistry has also gained popularity in the area of modification polymers due to its high selectivity, lack of side products and no additional requirement of catalyst.

The efficiency and regiospecificity of these reactions can also provide well-defined crosslinking in the material. For example, Lin and coworkers utilized photoclick-based reaction to obtain a well-defined degradable crosslinked network. In their study, norbornene functionalized PEG based polymer was crosslinked with a dithiol containing crosslinker in the presence of UV light to form hydrogel (Figure 1.3). They investigated degradation of hydrogel through hydrolysable ester bonds by analyzing its elastic modulus in a time interval with rheometer and observed a decrease in elastic modulus of hydrogel over time [10].

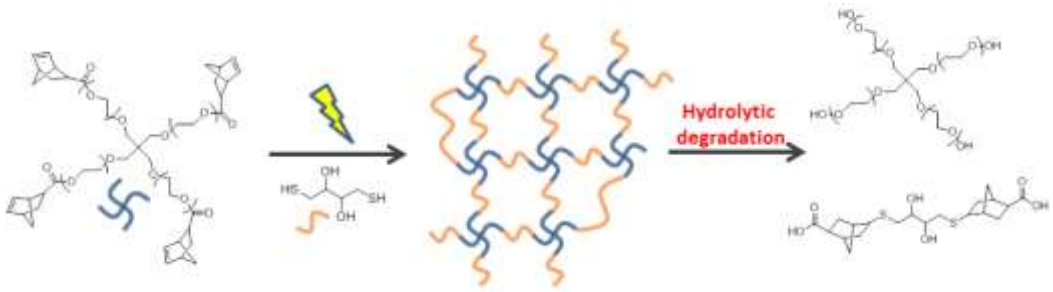

Figure 1.3. Hydrogel synthesis via thiol-ene reaction

In another example, Hilborn and coworkers reported a novel hydrogel system, where poly(vinyl alcohol) and linear poly(ethylene glycol)functionalized with pendant acetylene and azide groups functionalized were used to produce crosslinking via ‘click’ chemistry (Figure 1.4). Hydrogels fabricated using poly(vinyl alcohol) chain and linear PEGs cause difference in network properties in terms of inner structure of hydrogel. Instead of using linear PEG as crosslinker, for example, when using two different poly(vinyl alcohol) chains functionalized with acetylene and azide pendant groups

provides higher gelation efficiency and materials possess higher mechanical strength. Reason for this difference is because polymer chains are closer to each other when side chain functionalized polymers are employed [11].

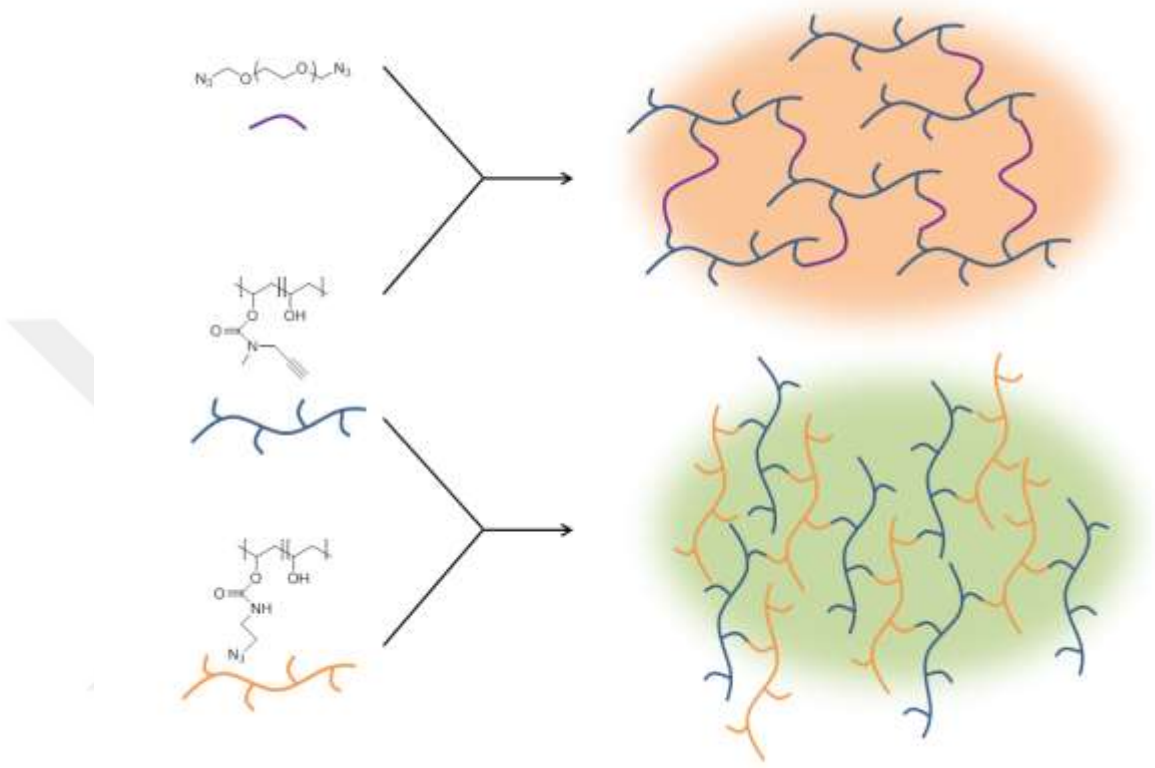

Figure 1.4. Hydrogel synthesis from polymer-crosslinker and polymer-polymer conjugates via click chemistry

1.2.1. Diels-Alder Click Chemistry

Diels-Alder (DA) reaction is a highly selective [4 + 2] cycloaddition between a diene and a dienophile that is highly accelerated in water (Figure 1.5) [12]. Furthermore, the DA click reaction is free from side reactions, and often does not require initiators, catalysts or coupling agents. Also, the reaction is reversible in nature i.e. the covalent bonds between a dienophile and a diene can be easily broken down by heating through retro-Diels-Alder (retro-DA) reaction. Over time or upon heating they can recombine to form the cycloadduct. Hence, materials that are fabricated via DA chemistry can be

classified as thermally-responsive materials. This reaction has been widely used to obtain self-healing materials due to this thermally-responsive character [13][14][15][16][17]. Wudl and coworkers reported materials that are crosslinked through DA reaction with excellent self-healing properties. In their study, furan and maleimide bearing polymers or cross-linkers were used to obtain remendable materials [18]. Similarly, Haddleton and coworkers reported polymers which have self-healing properties. In their studies, initiators and monomers which contain furan and maleimide cycloadduct were used to obtain crosslinked polymers. Unfortunately, requirement of high temperature for retro-DA reaction limits its application areas, furthermore it make impossible to use reversible properties under physiological conditions.

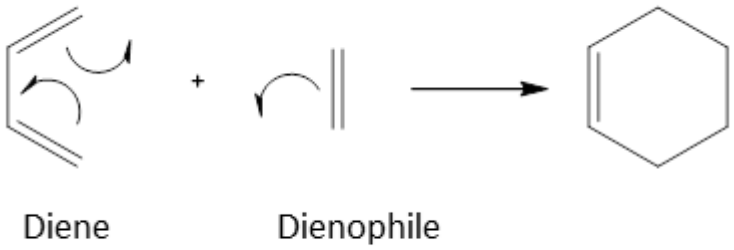

Figure 1.5. Diels-Alder cycloaddition reaction

In recent years, DA reaction has been utilized as a click reaction of choice of synthesis of hydrogels[19][20]. Recently, for example, Goepferich and coworkers made a number of contribution to Diels-Alder reaction based hydrogels [21][22][23]. They achieved fabricated maleimide and furan functionalized multi-arm PEG polymers to synthesize hydrogels (Figure 1.6.). They also investigated the stability of maleimide group in various conditions because maleimide group may undergo hydrolysis under aqueous conditions.

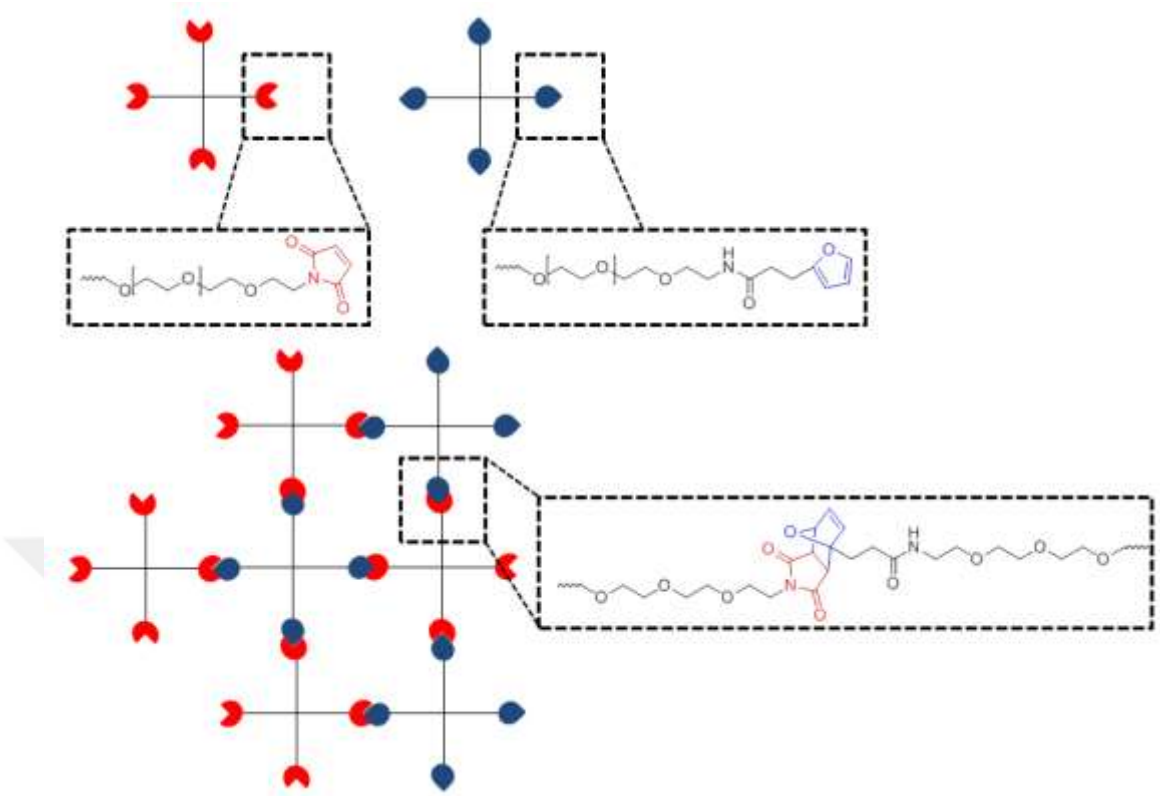

Figure 1.6. Synthesis of hydrogel with multi arm PEG polymers through Diels-Alder click reaction

One advantage of DA click chemistry over other click reactions is their efficiency in aqueous media under mild conditions. Several studies in the literature demonstrate that the efficiency of cycloaddition can be dramatically improved by using water as a reaction media [24][25][26]. For example, Marra and coworkers reported fabrication of a hydrogel system through aqueous Diels-Alder chemistry. In their study, furan and maleimide functionalized hyaluronic acid derivatives were synthesized to fabricate hydrogels in aqueous media. They also investigated rheological properties, where they observed a decrease in compressive modulus due to degradation of hydrogel and they demonstrate sustained protein release from hydrogels [27]. In a recent study, Schoichet and coworkers reported hyaluronic acid (HA) and PEG based hydrogels system by using Diels-Alder reaction. Furan functionalized HA were crosslinked with bis-maleimide PEG polymers in aqueous media. Their system demonstrated excellent cyto-compatibility with cell survivals rate > 98% after 14 days in culture. In another example, Wei and coworkers indicated that

there is a dramatic influence of solvent in the synthesis of hydrogel by Diels-Alder reaction. They synthesized furan bearing polymers by using *N*-vinyl-2-prrolidine and furfuryl methacrylate via AIBN initiated free radical polymerization [28]. Hydrogels were fabricated by crosslinking furan bearing polymer and PEG based bis-maleimide crosslinker (Figure 1.7.). They demonstrate that time of gelation depends on the number of furan group on the polymer chain, the reaction temperature and the solvent.

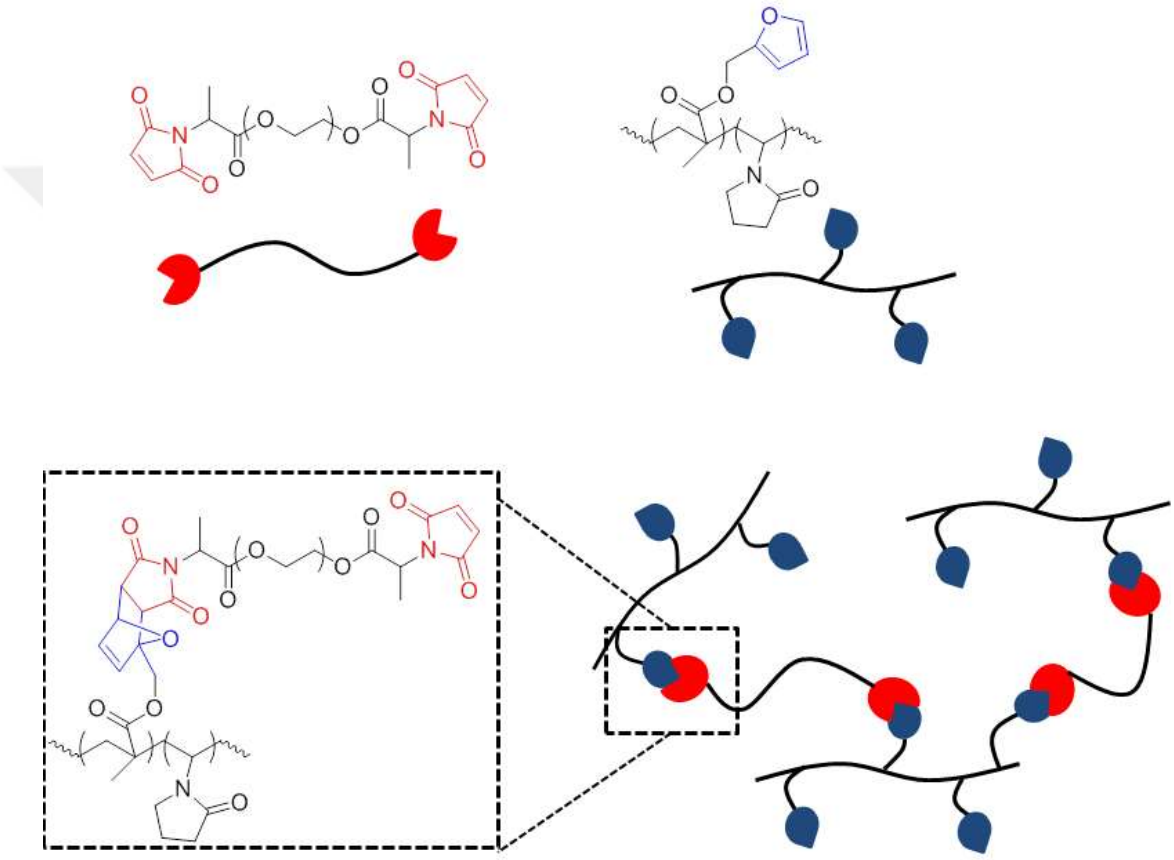

Figure 1.7. Hydrogel synthesis via Diels-Alder click reaction

In a series of reports, Singha and cowerkers have demonstrate that furan bearing polymers and copolymers that combined with methylmethacrylate can be stythesized via ATRP and RAFT and can be used to fabricate remendable films when combined with bis-maleimide linkers [29][20].

1.3. Single Electron Transfer Living Radical Polymerization (SET-LRP)

SET-LRP is known as a robust technique for ultrafast synthesis of ultrahigh molecular weight poly(methacrylate)s. Present mechanism of SET-LRP propose that it is catalyzed by electron-donors such as Cu(0) powder or Cu(O) wire in the combination with N-ligands such as Me_6Tren or PMDETA, and solvents that help rapid disproportionation of Cu(I)Br into Cu(0) activator and Cu(II) deactivator (Figure 1.8). Perfect retention of chain-end functionality of polymers has made SET-LRP an attractive technique for the synthesis of block copolymers, star polymers, copolymers, or the polymers with complex architectures [30]

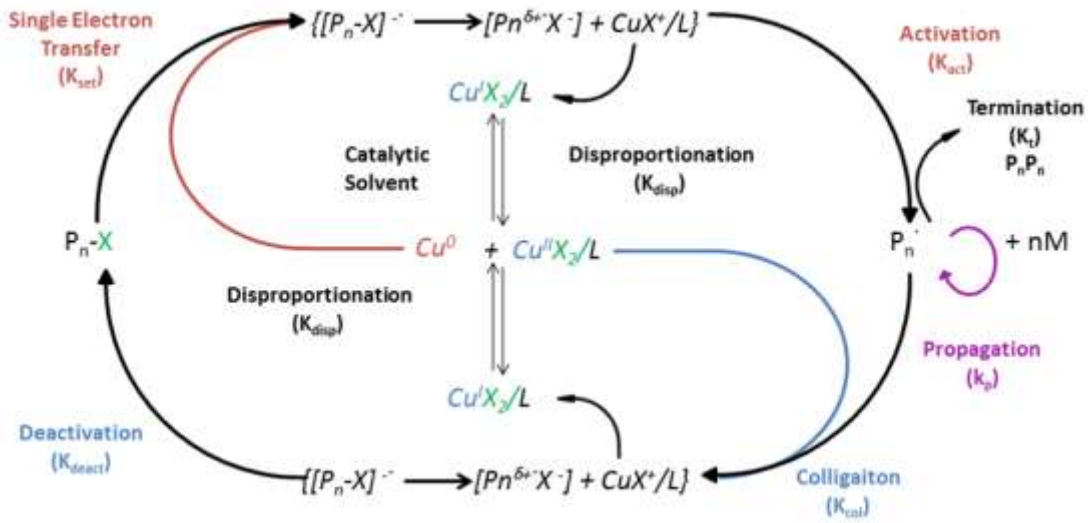

Figure 1.8. Mechanism of SET-LRP

Percec and coworkers reported polymerization of poly(ethylene glycol) methyl ether methacryalte in the absence and presence of air via SET-LRP. Kinetic studies of polimerization that were carried out in water and water-DMSO mixture combined with hydrazine-activated Cu(0) wire demonstrate that polymerization is controllable [31].

1.4. Stimuli Responsive Hydrogels

Over the past two decades, synthetic hydrogels have drawn a lot of attention not only because of their unique properties but also because of their potential uses in biomedical devices. Esspecially, hydrogel systems termed as ‘smart’ systems can be designed for spesific application. Stimuli responsive units can be integrated into the hydrogels system to can add unique properties to these materials. Chemical signals, such as pH, metabolities and ionic factors can change the molecular interactions or break chemical bonds. Physical signals, such as temperature or electrical potential also will effect the chemical interactions in the system. These interactions will alter properties of polymeric material such as solubility, swelling, redox state and crystalline/amorphous transition [32].

Severel stimuli-reponsive triggers that have been used to facilitate degradation of hydrogels at physilogical conditions include UV-irradiation, enzymes, pH, temperature, redox potential and ultrasound. Among them, temperature and pH responsive hydrogels have been pursued most widely, because both factors are physilogically important [33][34]. For example, Lee and coworkers reported a temperature and pH responsive hydrogel system. They incoporated temperature-sensitive poly(N-isopropylacrylamide) and pH reponsive alginate into the hydrogel system and this provides a change in the inner structure ınder specific environments. In this way, swelling and deswelling ratios of porous hydrogels system could be adjusted [35].

In recent years, redox responsive materials have drawn a lot of attention. Disulfide bonds are one of the most common redox responsive stimuli triggers. Disulfide bonds are known to be degradable bonds in the presence of reducing agents such as dithiothreitol (DTT), L-cycteine and glutathione through thiol-disulfide exchange reactions [36]. Recently, Du Prez and coworkers used this strategy to synthesize cryogels that could be degraded with considerable amount of reducing agent (Figure 1.9). Their studies shows that thiol exchange strategy is an efficient way to degrade bulk hydrogels [37].

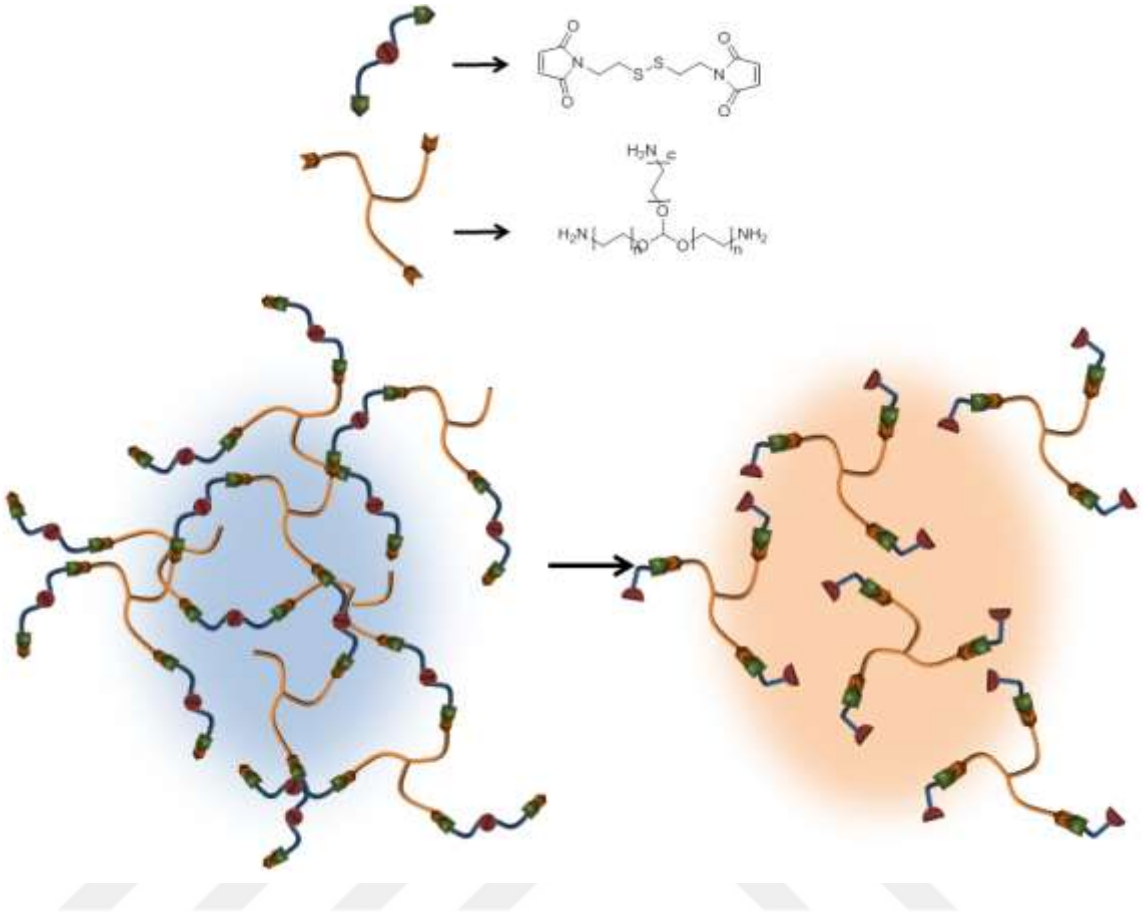

Figure 1.9. Redox responsive cryogel degradation in the presence of reducing agent

1.5. Rheological Analysis

Studying mechanical properties of hydrogels are important because mechanical strength of hydrogels can be adjusted through appropriate use of a crosslinker agent or by changing chemical or physical combination of the formulation. Hydrogels can be used in the field of drug delivery, protein encapsulation or gene therapy and properties of hydrogels such as gelation time and equilibrium elastic modulus are extremely important for all these applications.

Rheology is a very useful technique for characterization of mechanical properties of hydrogels because analysis with rheometer is fast, sensitive, requires small amount of sample and it can detect differences in architectures such as degree of crosslinking, structural homogeneity and molecular weight. In spite of simplicity of analysis, it is

difficult to compare mechanical properties of different materials. Morrison and coworkers reported a protocol study that can help to keep the continuity between rheological protocols. These protocols are the time sweep, strain sweeps and frequency sweep tests that provide information about linear-viscoelastic mechanical properties of hydrogels. Main interest here is finding out the appropriate strain and frequency values for the time sweep analysis [38].

Crosslinking density is another parameter that can be analyzed by rheology. Giasson and coworkers reported the rheological monitoring of polyacrylamide gelation with different crosslink density and temperature. Their study demonstrates that even small difference in crosslink density or temperature can be detected by rheometer. For example in figure 1.10.A, six different hydrogels with different crosslink densities demonstrate six different elastic modulus and this shows that rheology studies can detect even small differences in crosslinking ratio [39].

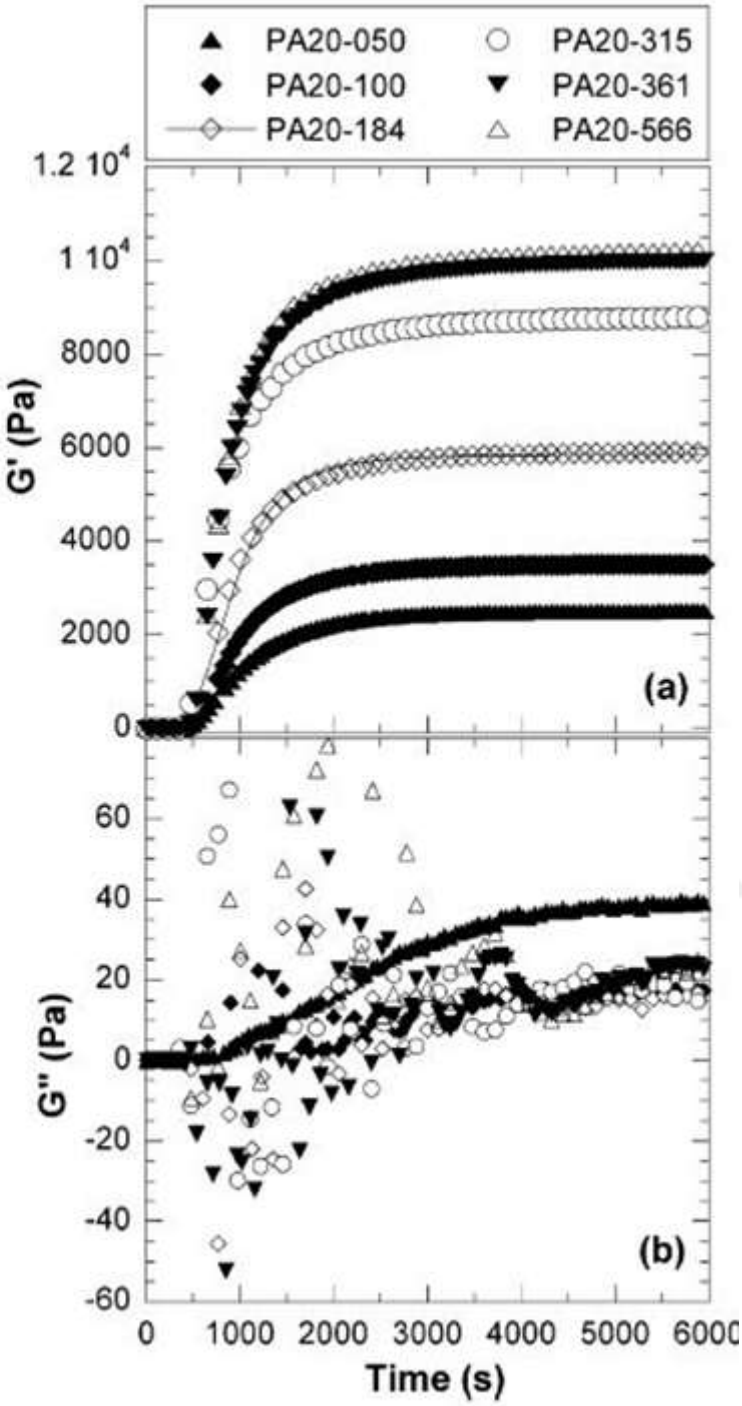

Figure 1.10. A sample of rheological analysis of a Hydrogel with different crosslinker concentration [39]

1.6. Protein Release from Hydrogels

Functions of proteins include catalysis of biochemical reactions, formation of membrane receptors, transport of molecules from a cell or an organ to another, and making the extracellular scaffold support. Hydrogels are crosslinked network of hydrophilic polymers that can absorb large amount of water without dissolving and this property makes hydrogels biocompatible because high water content and their soft nature is very similar to extracellular matrices. Their porous structure also provides a suitable environment to retain high amount of water soluble compounds like proteins. Many of the hydrogel formation procedures are very friendly in terms of protein stability because their formation can be carried out in aqueous media under mild conditions. In addition, proteins have limited mobility in hydrogels because they are generally big molecules and there longer residence time within the hydrogel matrix is favorable for protection of proteins 3D structure. These properties of hydrogels have led to their use as 3D scaffolds for proteins delivery through their controlled release in surrounding tissue.

Proteins can be loaded in the hydrogel matrix physically and release of proteins can be controlled with several methods such as diffusion, swelling, degradation or combination of these. For example, Schmidt and coworkers reported a study that 4-armed PEG base polymer and modified hyaluronic acid as a natural polymer are used to synthesize a network. They studied protein release from hydrogels via diffusion but not with degradation. As component of hydrogels, polymers and crosslinker are tunable materials in terms of chemical structures and that provide variety of design which change release profile. Leach and coworkers reported a hydrogels system which is hydrolytically cleavable for protein release. They observed protein release from hydrogels till 60 hours and complete degradation of the hydrogel [40]. Thus, design of hydrogel system and applied crosslinking strategies can be employed to create biodegradable scaffolds. If we define biodegradability as a conversion of material into water soluble end products that can be easily removed from the body, release of therapeutic proteins from biodegradable hydrogels will become a very attractive platform.

Yang and coworkers investigated the effect of method of polymeric network synthesis on protein release. Hydrogel systems were synthesized with polymers via step-growth polymerization and chain-growth polymerization separately. They deduced that length of PEG polymers synthesized via chain-growth polymerization, does not affect the protein release from hydrogel but decrease in PEG polymer concentration during gelation increased the protein release as expected [41]. Lie and coworkers also investigated the effect of diffusion and degradation of hydrogel on protein release. Multiblock poly(ether ester urethane)s consisting of poly[(R)-3-hydroxybutyrate] and poly(ethylene glycol) based hydrogel were used to study sustained release of bovine serum albumin for over 70 days; and they demonstrate that the first stage of release was due to diffusion and later stage was dominated by degradation of hydrogel (Figure 1.11.) [42].

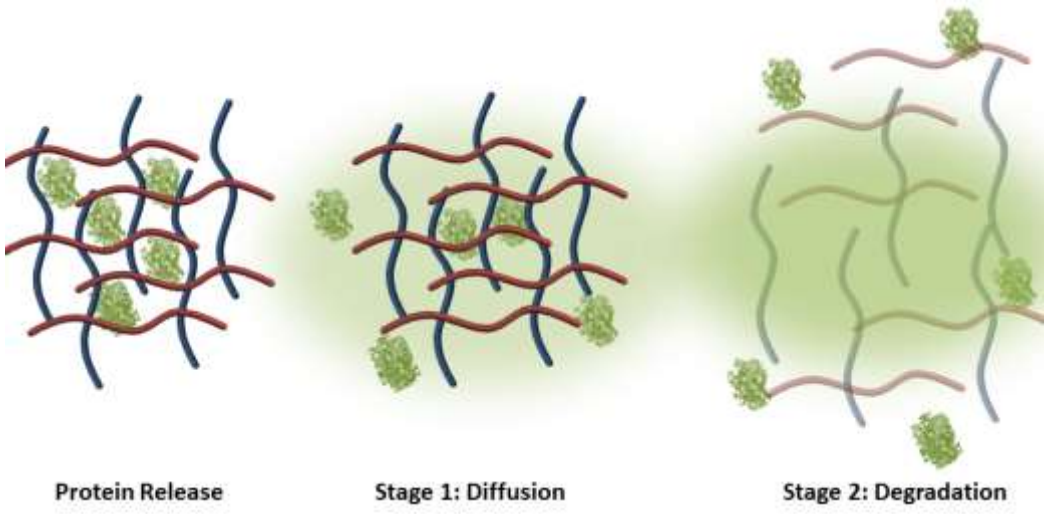

Figure 1.11. Diffusion and degradation depended protein release

Censi and coworkers reported effect of the molecular structure of the polymer on protein release. They fabricate hydrogel via photopolymerization of poly(N-(2-hydroxypropyl)methacrylamide lactate) with various amount of lactide and methacrylate groups and poly(ethylene glycol) with different molecular weights. They deduced that decreasing in the PEG's molecular weight and increase in number of methacrylate group on the polymer led to higher crosslink density and to stiffer hydrogels. More importantly they succeeded to tune bovine serum albumin (BSA) release by using different polymer combinations and they managed release of BSA up to 2 months [43].

2. AIM OF THE STUDY

Aim of this study is to synthesize novel degradable hydrogels using DA reaction for adjustable protein release through integration of redox responsive degradation trigger. Bulk hydrogels can be prepared with furan bearing polymer and disulfide containing bis-maleimide based crosslinker via the Diels-Alder reaction (Figure 2.1.). Unreacted furan groups in the hydrogels can be used to further functionalize with maleimide containing molecules. Incorporation of disulfide bonds into the crosslinker allows degradation of hydrogels with reducing agents. Protein encapsulation can be accomplished under mild conditions their release can be controlled via tuning the degradation rate of hydrogels.

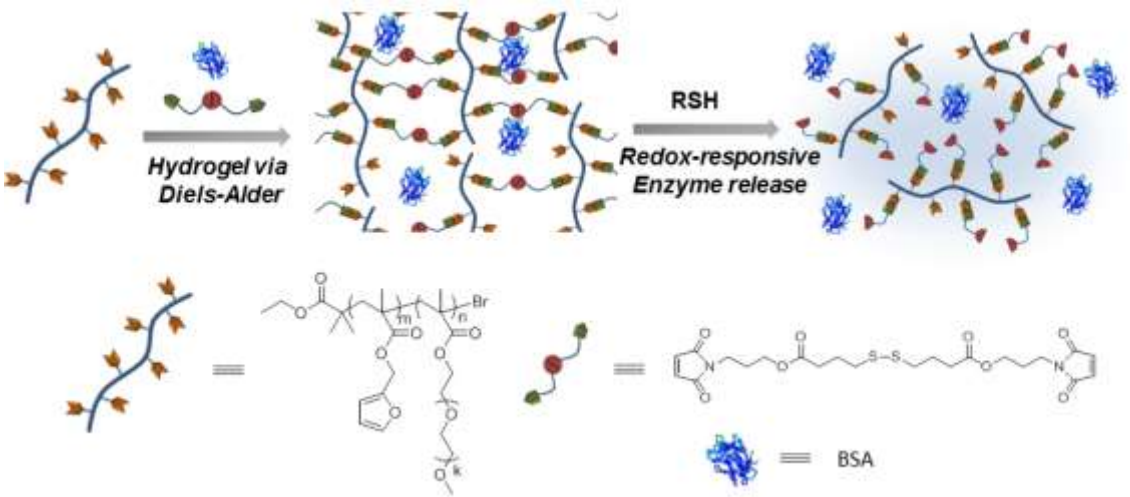

Figure 2.1. General scheme of the synthesis and degradation of protein loaded hydrogels.

3. RESULTS AND DISCUSSIONS

3.1. Synthesis and Characterization of Polymers

In recent years, polymers containing furfuryl methacrylate have been synthesized using ATRP and RAFT [37][19]. Recently, single electron transfer living radical polymerization (SET-LRP) has become a widely used polymerization technique due to its fast polymerization rate, yielding color free polymers with predictable molecular weight at room temperature with very good chain-end functionality [24]. In our study towards fabrication of hydrogels, furfuryl methacrylate was used with the hydrophilic monomer poly(ethylene glycol) methyl ether methacrylate (PEGMEMA) to obtain reactive copolymers. It is expected that such polymers with functional side chains may provide a good control of mechanical properties in fabrication of hydrogel since the number of pendant furan group on the chains can be controlled via SET-LRP. Briefly, our study demonstrates that mechanical properties of hydrogels directly affect the protein release profiles and mechanical properties of hydrogels are directly related to number of crosslinked installed using pendant furan groups. To explore polymerization of FuMA with SET-LRP, a series of polymers (P1-P5) were synthesized with different molar ratio of FuMA and PEGMEMA. Summary of homopolymerization and copolymerization conditions and results are shown in Table 3.1. The experimental ratio of the incorporated monomers was calculated by integrating the peak intensities from FuMA and PEGMEMA in the ^{1}HNMR spectrum (Figure 3.2). Integration of furan bearing monomer peak at 7.4 ppm and integration of PEGMEMA peak at 3.3 ppm was used to determinate the copolymer compositions. For example, according to ^{1}HNMR spectrum, ratio of FuMA to PEGMEMA was 1 to 2.5 on a single chain. GPC results exhibit number of average molecular mass of copolymer P3 which was used for all gelations was around 8500 $gmol^{-1}$ with molecular weight distribution of 1.2.

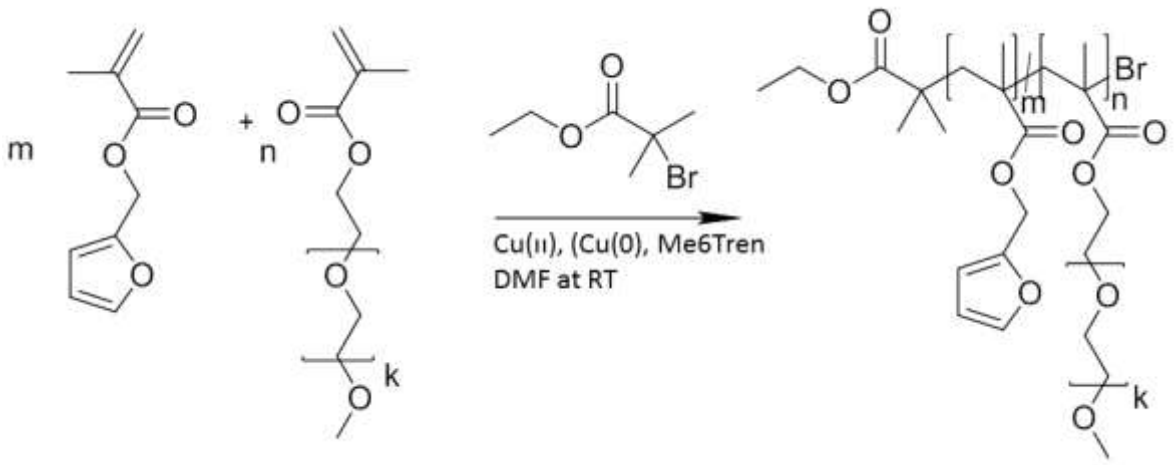

Figure 3.1. Synthesis of copolymer of FuMA and PEGMEMA

Table 3-1. Properties of homopolymers and copolymers obtained by SET-LRP of FuMA and PEGMEMA

Polymer [c]	$[M]_1/[M]_2$ [b]	$[M]_1/[M]_2$ [c]	M_n [g/mol^{-1}] [d]	M_w/M_n [e]	Conv.[%]
P-1	1/0	1/0	7100	1.4	65
P-2	0/1	0/1	12000	1.3	93
P-3	1/2	1/2.5	8500	1.2	71
P-4	1/4	1/2.7	8100	1.2	70
P-5 [f]	1/4	1/4.3	5000	1.1	72

[a] $[I]:[M]_{total}:[CuBr_2]:[Me_6Tren]$:1:10:0.05:0.12
[b] Theoretical mol equivalent; $[M]_1$: FUMA, $[M]_2$: PEGMEMA(300)
[c] Calculated mol equivalent by ^{1}H NMR; $[M]_1$: FUMA, $[M]_2$: PEGMEMA(300)
[e] Estimated by GPC eluted with THF
[f] $[M]_2$: PEGMEMA(500)

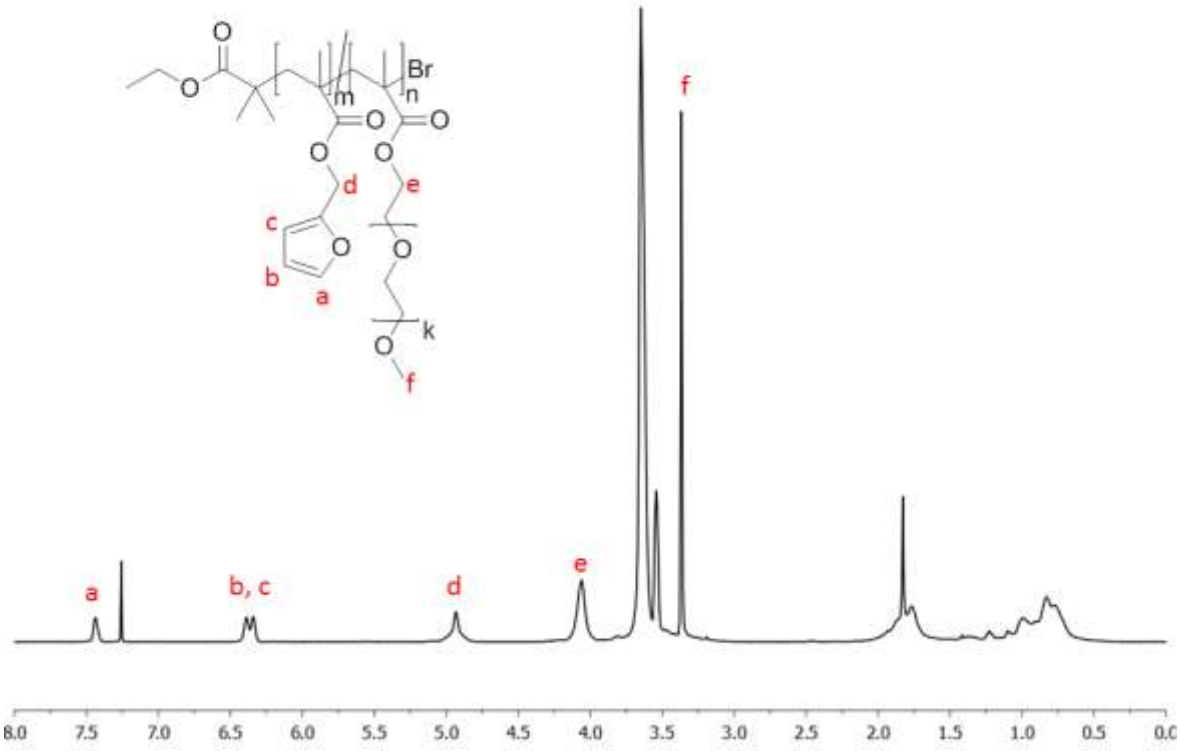

Figure 3.2. ^{1}H NMR spectrum of copolymer of FuMA and PEGMEMA

3.2. Synthesis and Characterization of Bis-maleimide Crosslinker

A disulfide containing bis-maleimide crosslinker was synthesized to install redox-responsive cross-linkages. First, 4,4-‘dithiodibutyric acid was functionalized with furan protected maleimide bearing alcohol in the presence of EDCI and DMAP via esterification reaction (Figure 3.3.). Unprotected maleimide groups were obtained using retro Diels-Alder reaction. Retro Diels-Alder reaction unmasked the maleimide unit completely as deduced by disappearance of the 2 peaks at 6.4 and 4.9 ppm (Figure 3.4.) and appearance of a new peak at 6.7 ppm in the product which belongs to the vinylic proton on the maleimide (Figure 3.5.) and was accompanied with disappearance of peaks at 6.5 ppm and 5.25 ppm which belongs to furan protection group. Additionally, ^{13}C NMR analysis was also used to confirm the structure of thus obtained crosslinker (Figure 3.6.).

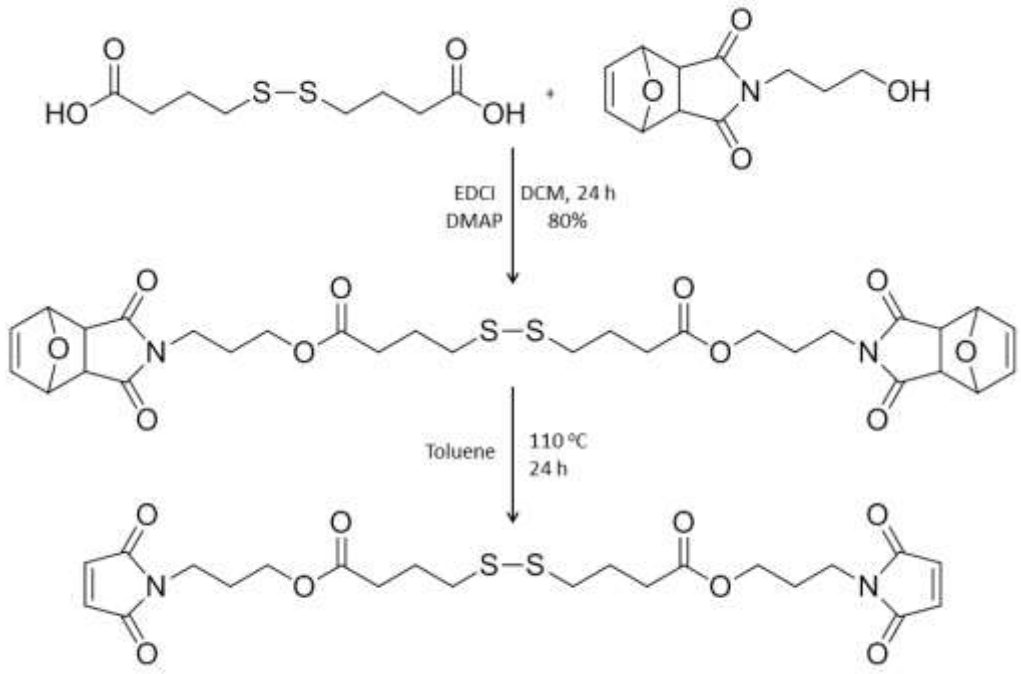

Figure 3.3. Synthesis of bismaleimide crosslinker

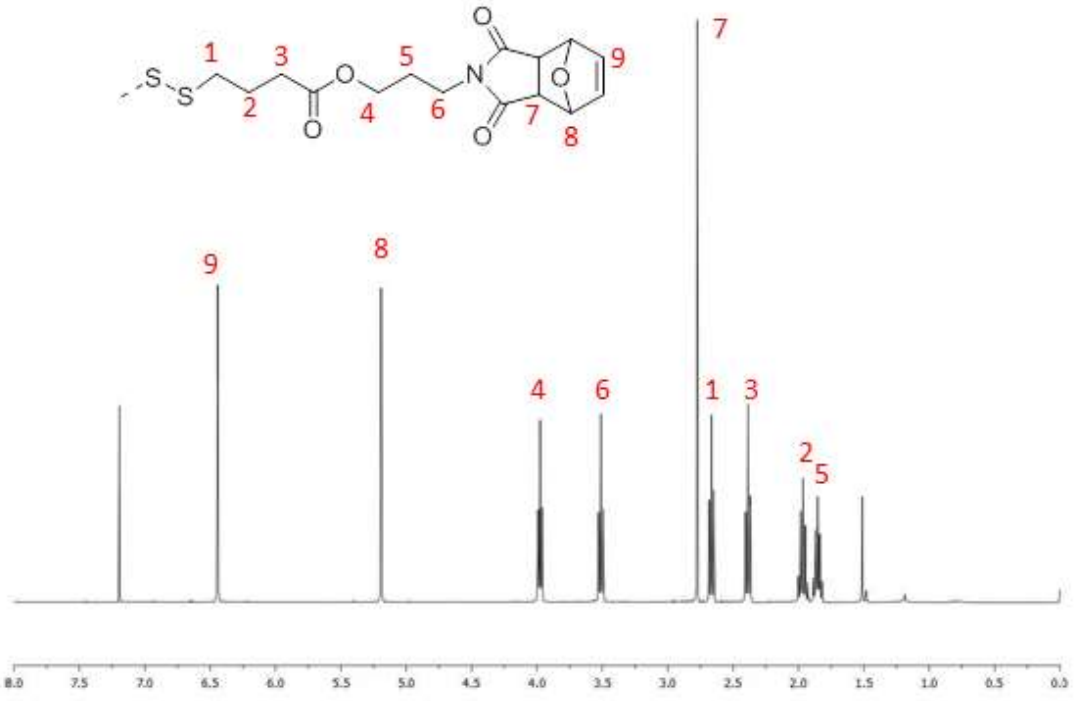

Figure 3.4. ^{1}H NMR spectrum of protected bismaleimide crosslinker

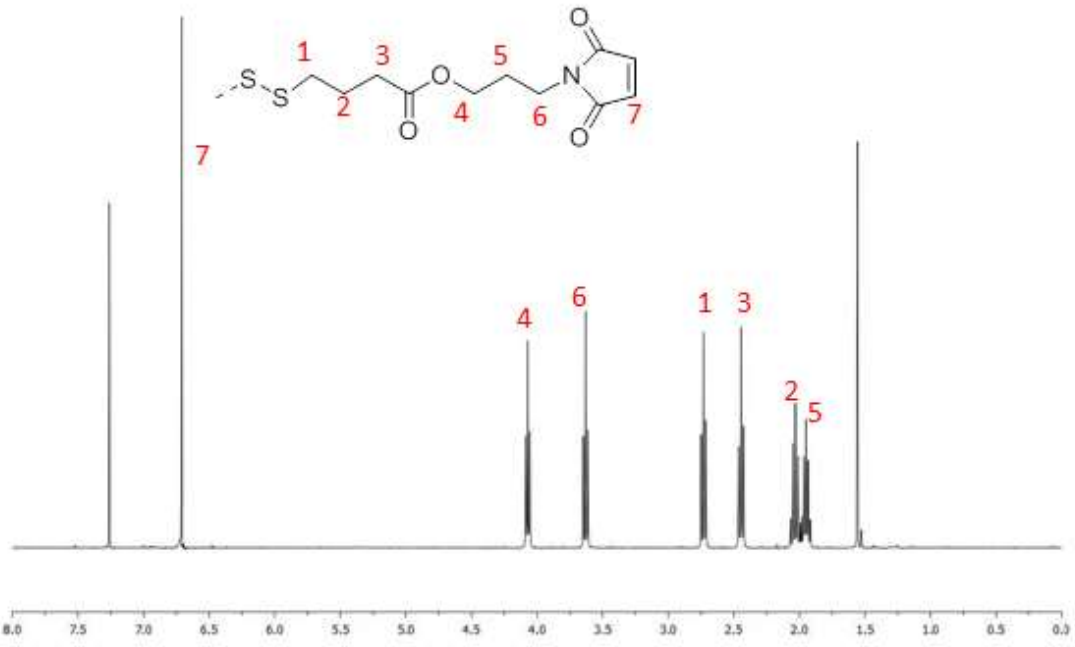

Figure 3.5. ^{1}H NMR spectrum of bismaleimide crosslinker

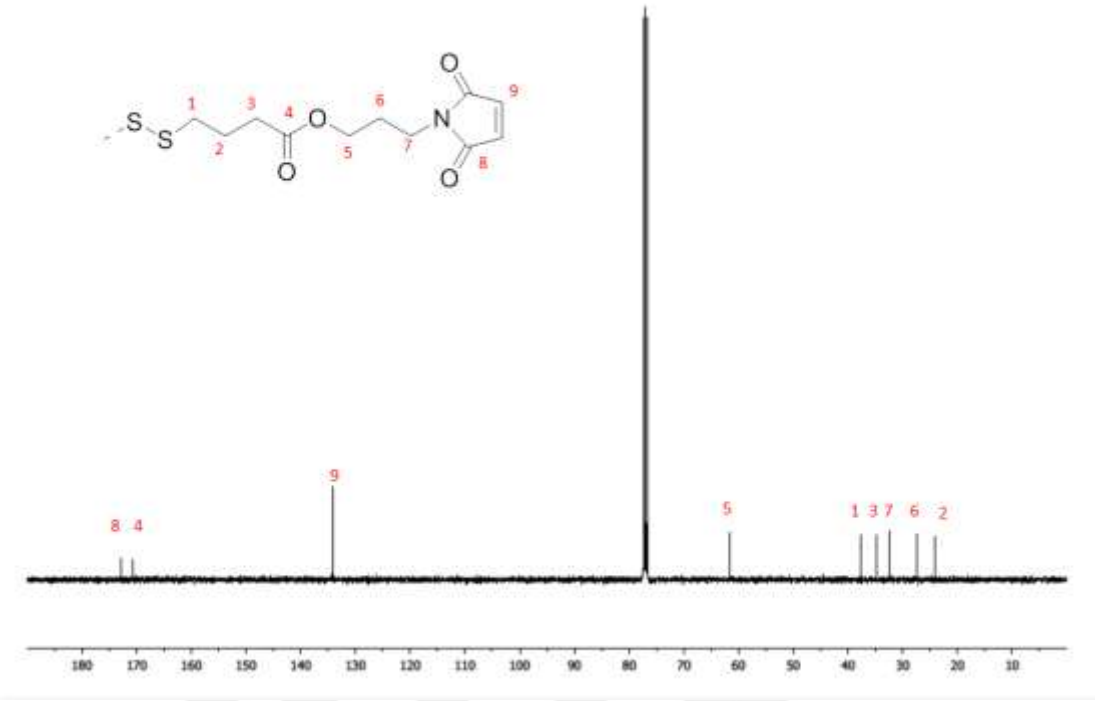

Figure 3.6. ^{13}C NMR spectrum of bis-maleimide crosslinker

Kirchhof and coworker utilized the hydrolytic stability of 8arm-PEG10k-maleimide. It is well known that in the literature, maleimides undergo hydrolysis in alkaline solution but until this study there was no data for this degradation. They show that maleimides of 8armPEG10k hydrolyzed more in alkaline solutions the acidic solutions by analyzing maleimide peak in UV spectrometry [21]. In our study, stability of maleimide group becomes an important point due to long gelation times. For that reason, bis-maleimide crosslinker was analyzed via UV spectrometry against time in PBS and 20% PBS-DIOXANE mixture. We found that maleimide are stable in PBS-DIOXANE mixture but crosslinker undergoes hydrolysis in PBS solution in the range of gelation time (Figure 3.7.).

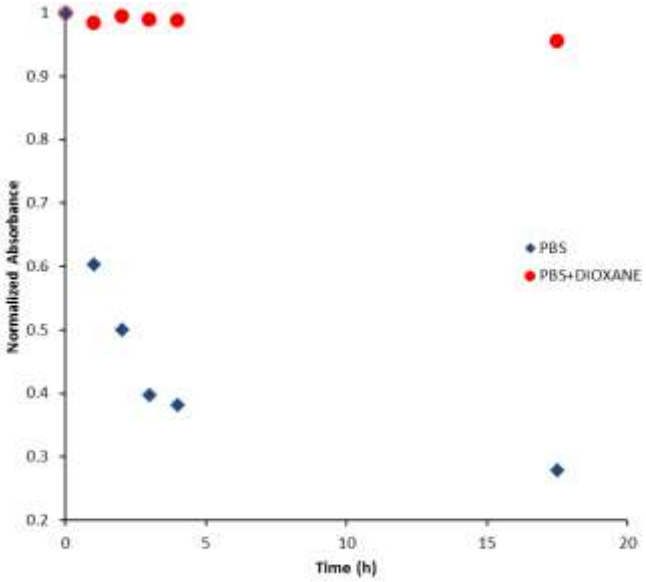

Figure 3.7. Hydrolytic stability of bis-maleimide crosslinker

3.3. Preparation and Characterization of Hydrogels

A series of hydrogels (HG1-HG3) was synthesized with different ratios of maleimide groups to number of furan groups on the polymer (Table 3.2.). Hydrogels were prepared using a simple process that involved mixing of the polymer and crosslinker in a water/dioxane (3:1) mixture at different temperatures (Figure 3.8.). Thus synthesized hydrogels were transparent and stiff at room temperature. The hydrogel compositions and conversions of gelation are summarized in Table 3.2. Results indicate that increment in crosslinker density leads to higher conversions, as expected. Furthermore, hydrogels which have different crosslinker densities exhibit differences in various physical properties such as swelling, mechanical properties and stability. Rheology analysis of hydrogels with various crosslink density exhibits that more crosslinked hydrogel has higher G^I value which represent stiffer hydrogels and stiffness of hydrogels can be reduced by using less amount of crosslinker (Figure 3.9.A). Swelling degree of hydrogels can be adjusted by changing the amount of crosslinker involved in the gelation. Figure 3.9.B shows that decrease in the crosslinker density dramatically increased the swelling degree of the hydrogels. Mechanical properties of hydrogels are directly related to water uptake capacity of the networks. The study of the hydrogel series (Figure 3.9.) demonstrates that hydrogel

with high mechanical strength also possessed lower degree of swelling and a decrease in mechanical strength increases the degree of swelling of hydrogels. Also, the porous structure of hydrogels is directly related to swelling degree of hydrogels. From the SEM images of hydrogels (Figure 3.10.), it is clear that the porosity of hydrogels are decreasing with increasing crosslinker. Moreover porosity of hydrogels correlates with the swelling degree of hydrogels. For example, HG 1 is less crosslinked than HG 3; hence HG 1 is able to swell more water due to its porous structure. HG 3 has no porosity and low swelling degree and this is because of high crosslink density of HG 3.

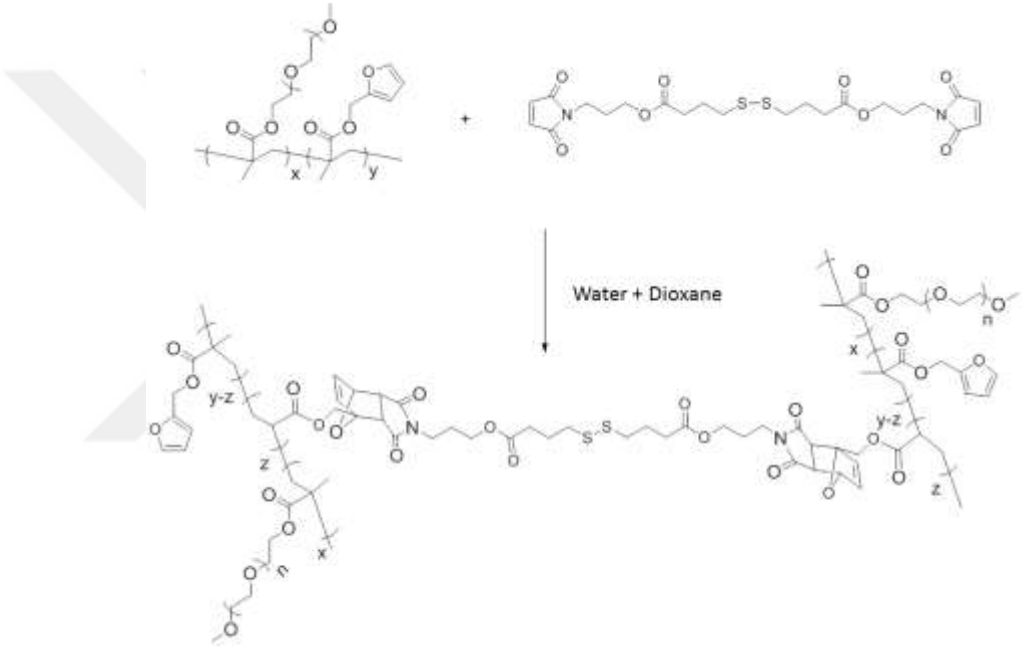

Figure 3.8. Gelation mechanism of hydrogels

Table 3-2. Library of hydrogels with different crosslinker density

Hydrogel	$[M]_1/[M]_2$ [a]	Conversion
HG 1	10/4	72
HG 2	10/6	77
HG 3	10/8	81

[a] Theoretical mol equivalent; $[M]_1$: FURAN, $[M]_2$: MALEIMIDE

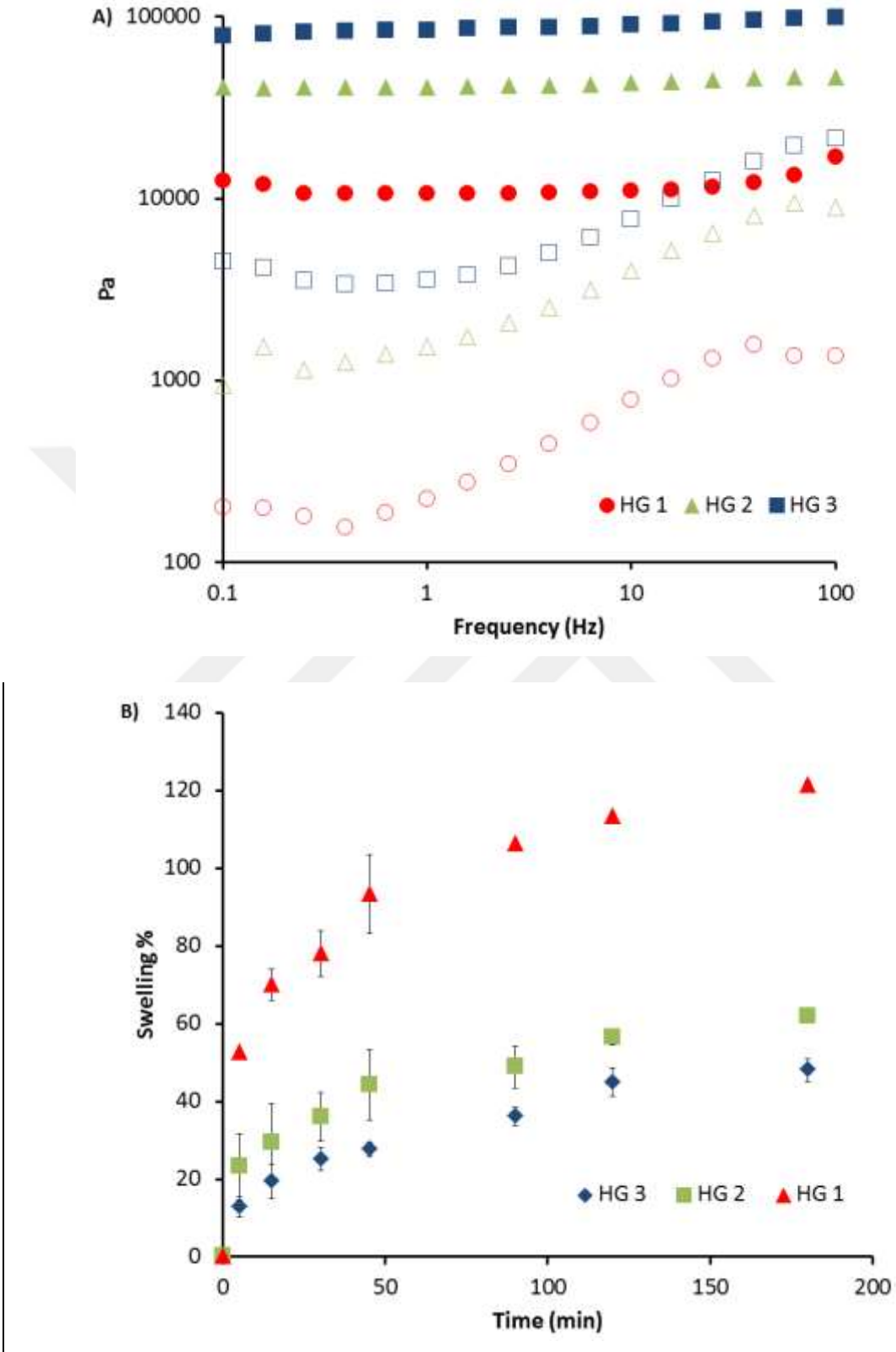

Figure 3.9. A) Frequency sweep test of HG 1, HG2, HG3, (empty square: G^{l}, empty triangle: G^{ll}) B) Effect of crosslinker density on swelling degree of hydrogels.

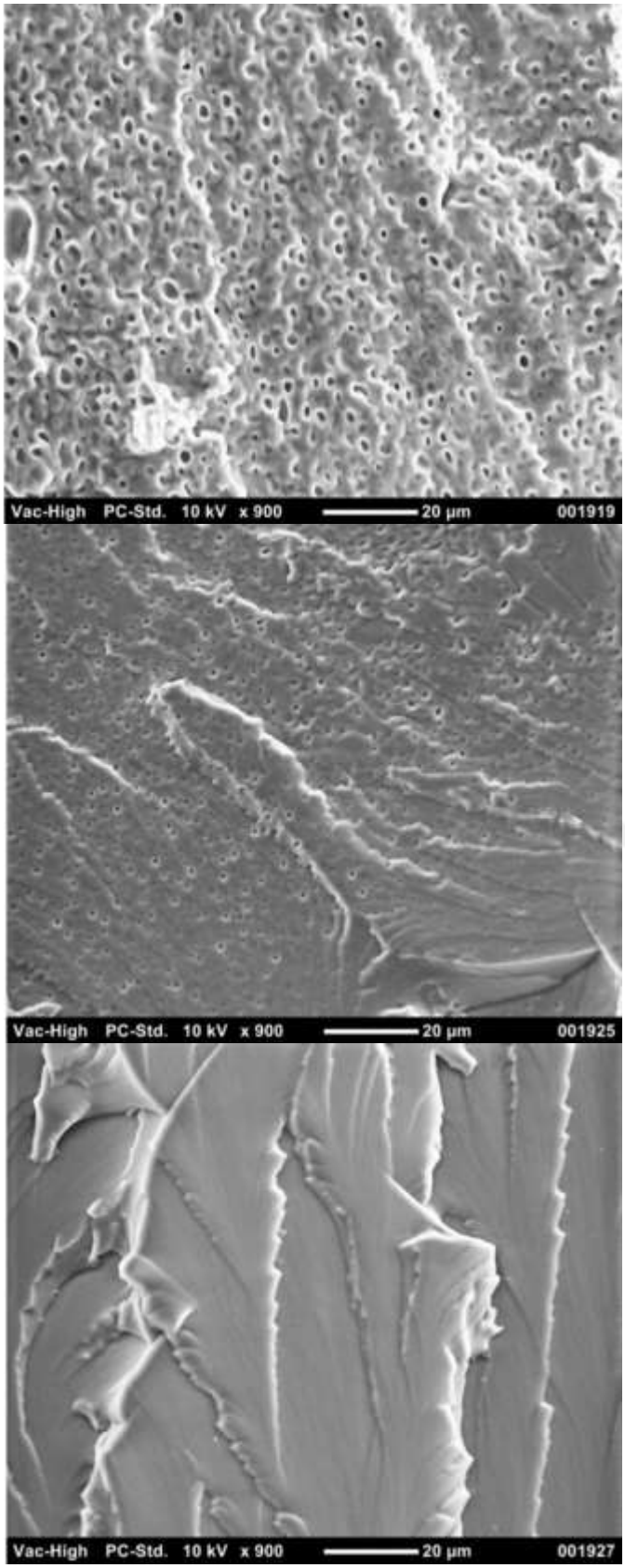

Figure 3.10. SEM images of HG 1 (Top), HG 2 (middle), HG 3 (bottom)

3.4. Rheological Analysis of Hydrogels

Strain sweep test is recommended for the control linear viscoelastic (LVE) limits of hydrogels[38]. Strain sweep test from 0.01 to 100 strain was applied on the hydrogel. Results shown in Figure 3.11, indicate that HG 2 showed a linear behavior G^1 for all strain values. Linear strain graph represents that hydrogel preserves its inner structure without any breakdown for all strain values. A small strain 0.5 was selected for further study against any structural weakness due to the degradation of hydrogels.

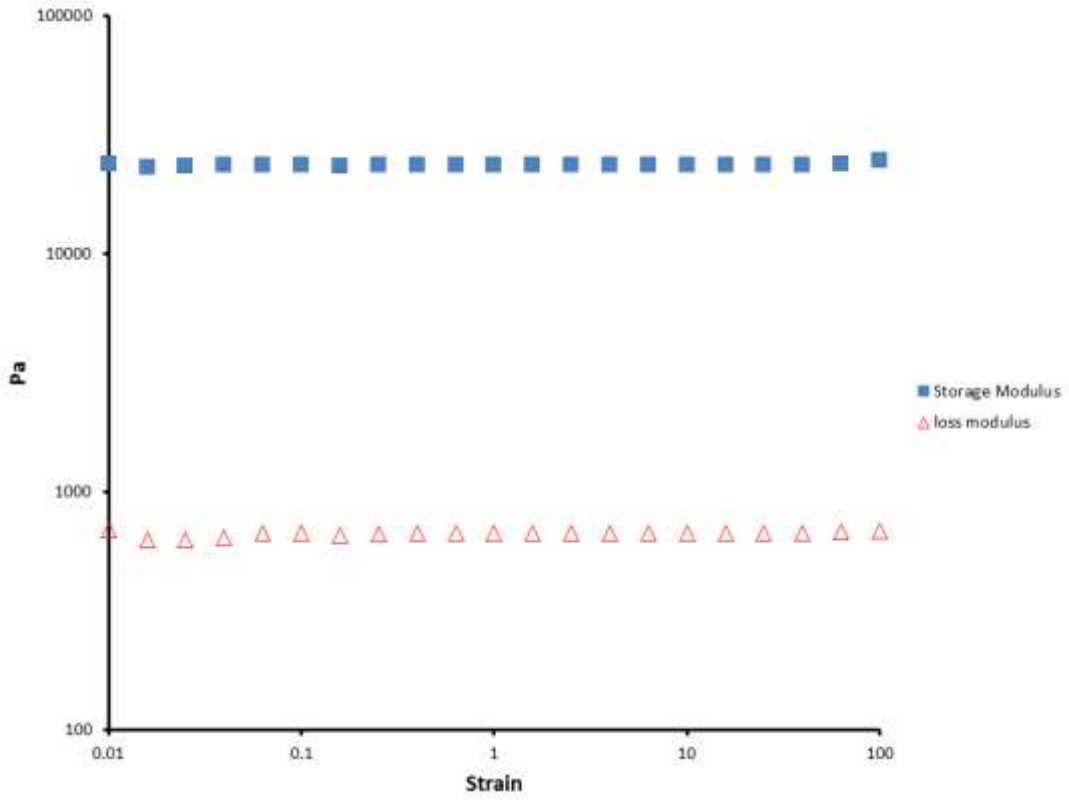

Figure 3.11. Strain sweep test for HG 2

In our study, we show that composition of the hydrogel is directly related mechanical properties of the hydrogel. Mechanical behavior of the hydrogel was measured by frequency sweep test from 0.01 to 100 Hz by using strain value of 0.5. The results of frequency sweep test shown in Figure 3.8.A. illustrates that all of the hydrogels reflected gel behavior and storage modulus of HG 3 is higher than the others. In summary, we can

say that higher crosslinker density makes hydrogel stiffer and decreases water uptake capacity. An oscillation frequency of 10 rad/sec was selected for future test based on good torque signal at that frequency [38]. Thus mechanical strength of the hydrogels can be tuned by varying the amount of crosslinker in the hydrogel.

Time sweep test was performed to follow gelation process, degradation process, gelation time and final elastic modulus value which are directly related to crosslinker density of hydrogels. Time sweep tests are performed by using strain 0.5 and frequency of 1.5 Hz. Storage modulus could not be observed at the beginning because the G^l value was very low. Just before gelation point storage modulus becomes noticeable in all time sweep tests. Gelation point is the crossover point of loss and storage modulus; and after gelation point, storage modulus starts to dominate over loss modulus which means that the material has a solid-like structure. Gelation process of HG 3 was studied at 25 ^oC, 30 ^oC and 37 ^oC to investigate the temperature effect on gelation. Briefly, we note that temperature directly affects the gelation process, due to the higher rate of Diels-Alder reaction (Figure 3.12.B). At 37 ^oC, gelation point of hydrogel is the shortest one and gelation point become much longer at 25 ^oC. Due to the fast gelation at 37 ^oC, a sharp increase in G^I value can be seen while G^I has a slower evolution at 25 ^oC.

Time sweep tests were also studied to investigate the effect of crosslinker density on gelation point and storage modulus. We desire to obtained hydrogels which have different physical properties that can used to tune the protein release profiles. For this purpose, gelation processes of three different hydrogel (HG 1-HG 3) were studied. As clearly seen in Figure 3.12.A. Gelation of HG 3 is much faster than the others due to the high crosslinker density. Amount of crosslinker also affect the stiffness of the hydrogels and highly crosslinked network also gives higher storage modulus.

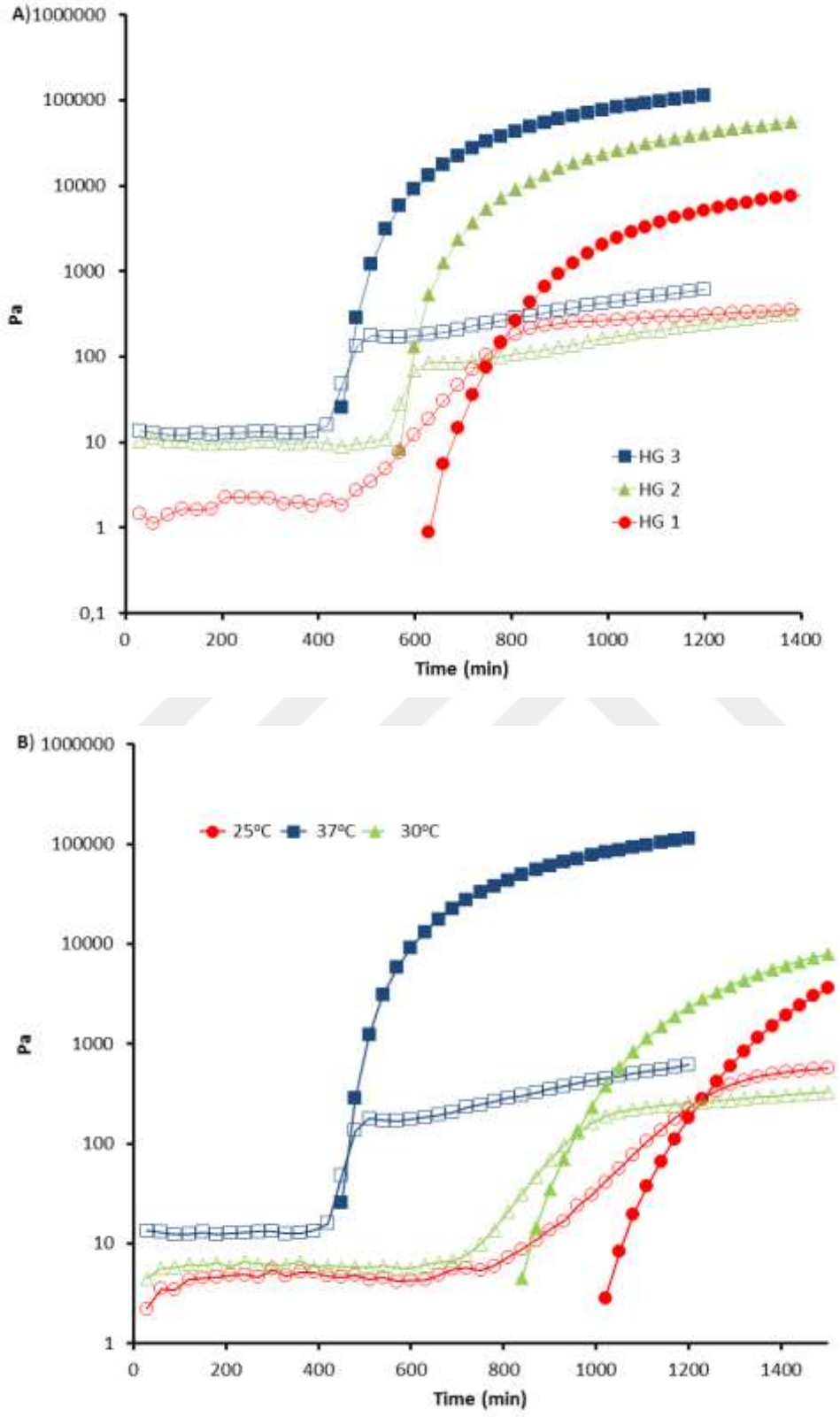

Figure 3.12. A) Time sweep test for hydrogels with different crosslinker density B) Time sweep test of HG 3 at different temperatures (empty square: G^{1}, empty triangle: G^{11})

3.5. Degradation of Redox Responsive Hydrogels

In our study, hydrogels contain redox responsive disulfide bonds and reversible Diels-Alder linkages at the same time. Although there may be some cycloadducts with *endo*-stereochemistry that have a relatively low cycloreversion temperature around 40 ^{o}C, these hydrogels were found to be quite stable under non-reducing conditions. It is well established that glutathione and DTT can easily reduce the disulfide bonds. Also, it is well documented that when dithiols like DTT is used as a reducing agent, disulfide cleavage is faster; and thus we utilized DTT to trigger this degradation so that we can study the process in a reasonable time scale. In order to compare the effect of rDA and disulfide cleavage on degradation, 80 mg of dry hydrogel (HG1) was placed in 3 mL of PBS solution and 3 mL of DTT containing (21 mM) PBS solution at 37 ^{o}C. The hydrogel in DTT solution lost its shape after 2 days while the hydrogel in PBS solution was still stable (Figure 3.13.). Rheological studies also demonstrate that the efficiency of DTT on degradation of hydrogels was much higher than the effect of rDA reaction (Figure 3.15.).

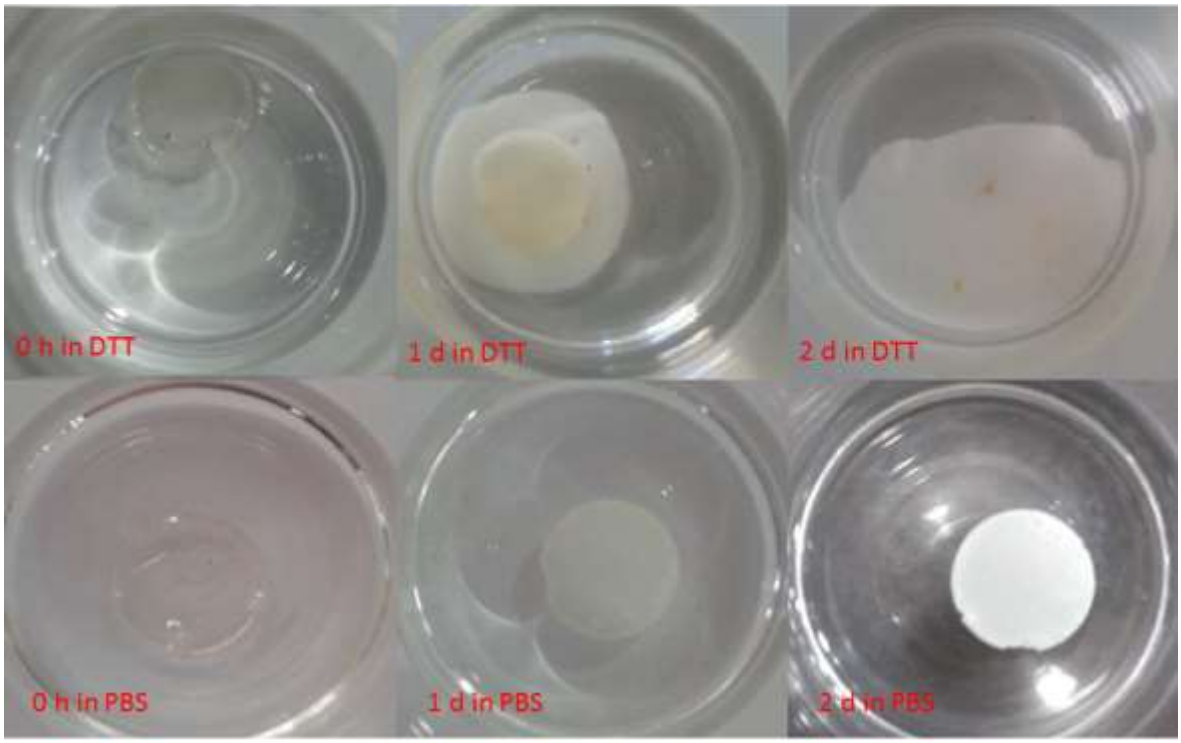

Figure 3.13. Degradation processes of HG 1 in PBS and DTT (21 x 10^{-3} M) solutions

3.6. Degradation Studies of Hydrogels

Degradation of hydrogels by cleavage of disulfide bonds was studied with time sweep test. Hydrogels lost their highly crosslinked structure in the presence of DTT and they lost their stiffness. Due to this degradation, storage modulus decreased and a crossover between G^{I} and G^{II} was observed (Figure 3.14.). Crossover is the point where hydrogel loses its gel structure and goes to the sol form. This complete transform in a feasible scan time could be seen only in extreme conditions which includes highly concentrated DTT solution (200 mM).

Behavior of the hydrogels in the PBS solution without DTT can be used as a control experiment. It is known that some hydrogels can be degraded due to the retro Diels-Alder reaction. However, in our cases, effect of degradation of the disulfide bonds in the presence of DTT was much higher than the effect of retro Diels-Alder reaction. The dominance of disulfide bond degradation on the retro Diels-Alder reaction was studied with rheometer. Figure 3.15 shows that mechanical properties of HG 1 in PBS solution do not change and are stable. In the beginning, an increase in storage modulus was observed due to the swelling then storage modulus becomes stable and no degradation can be observed. Mechanical response of the hydrogel in DTT solution was much more different. A sharp decrease can be seen in storage modulus because of the degradation of disulfide bonds. This difference comes up due to low hydrophilicity of the polymers that makes retro Diels-Alder reaction slower.

Results from rheological analysis prove that physical property of the hydrogels can be varied by changing amount of crosslinker and degradation can be triggered in the presence of DTT. As a next step, we probed if we can use these properties to control the release of biomolecules encapsulated within hydrogels.

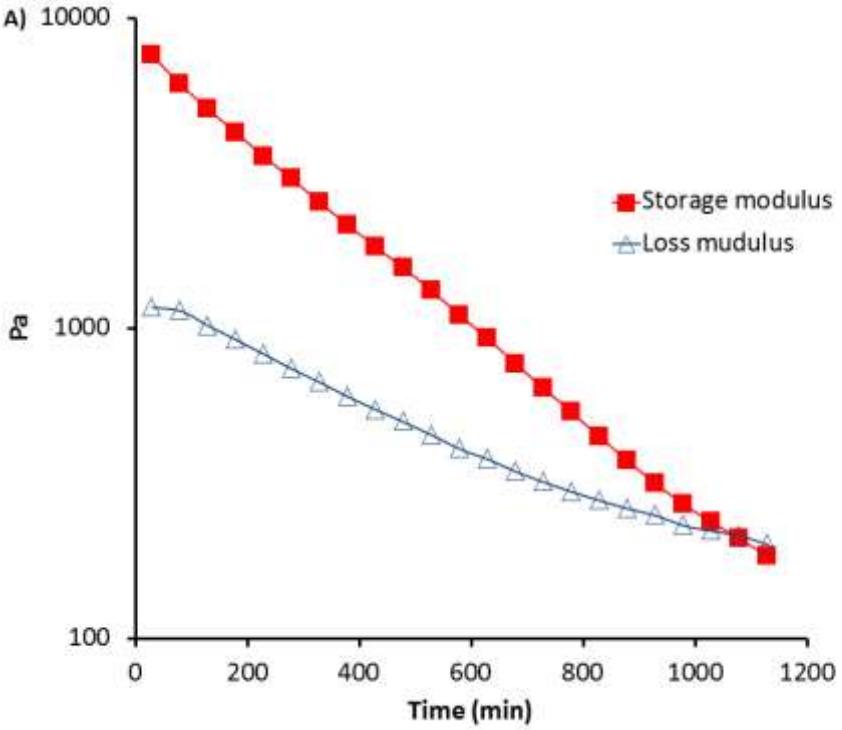

Figure 3.14. Rheological analysis of degradation process of hydrogels

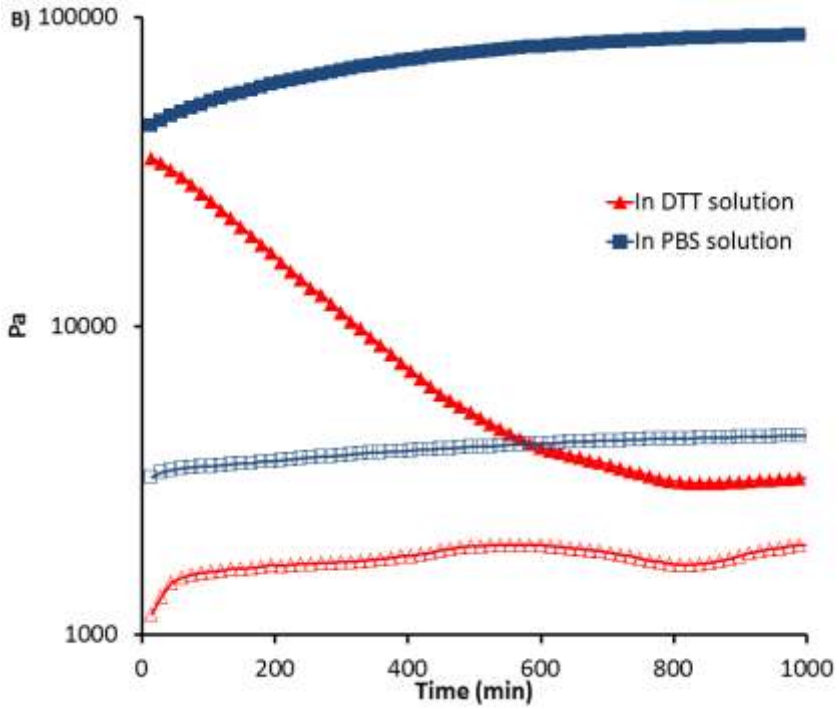

Figure 3.15. Degradation of HG 1 at 37 ^{o}C in PBS and DTT solutions.

3.7. Protein Release Studies

Hydrogels HG 1, HG 2, HG 3 were used for bovine serum albumin (BSA) release study. Fluorescent dye labeled BSA was loaded into hydrogels as described above. Un-encapsulated BSA molecules were washed with distilled water and analyzed with UV-Vis to calculate how much enzyme was trapped inside hydrogel. We probed fluorescein labeled BSA release with and without DTT. The released fluorescein labeled BSA dissolved in water and emitted green fluorescence under UV light. It was clear that in the presence of DTT, amount of BSA in the solutions was higher (Figure 3.16.A). Hydrogels (HG 1, HG 2, HG 3) were immersed in PBS and DTT solution (21 mM). At every analysis time point, solutions were completely changed with fresh ones and extracted solutions were measured with UV for quantifying the amount of released BSA. Two distinctive release characteristics were seen for hydrogels. Figure 3.16.B shows that release profile in PBS solutions and DTT solution are different from each other. Due to the disulfide bond cleavage in DTT solution, cumulative release of BSA was almost 6 times higher in HG 1. Also release profile can be correlated with degree of crosslinking. It is harder to degrade more crosslinked and stiffer hydrogels. BSA Release from HG 1 was 2 times higher than HG 2 due to less amount of crosslinking in the former hydrogel. These studies clearly highlight that protein release can be fine-tuned based on the amount of degradable crosslinking within the hydrogels.

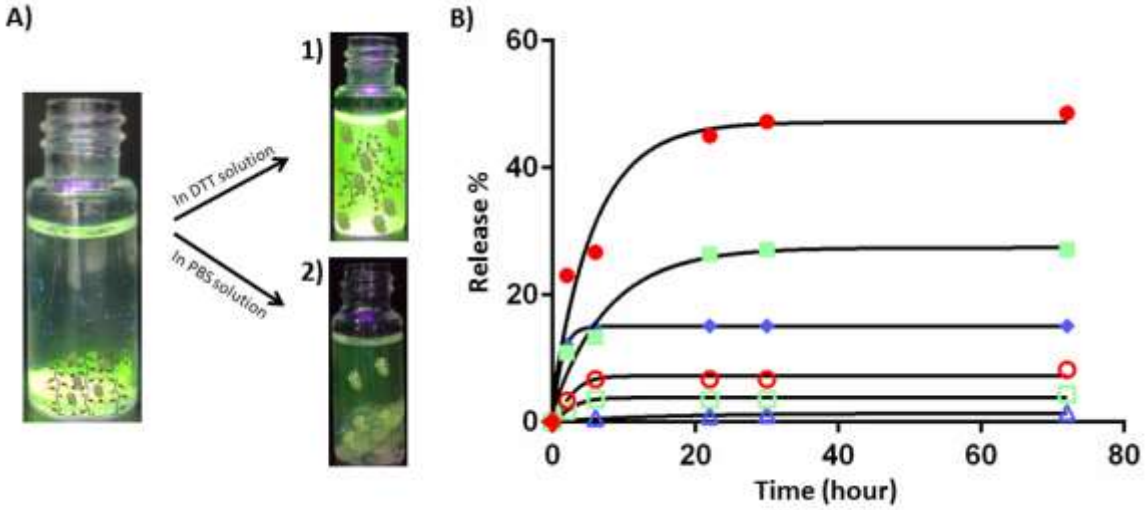

Figure 3.16. A) Release from BSA loaded degraded hydrogel in 21 mM DTT solution and in PBS solution. B) Release profile of HG 1 ( ) , HG 2 ( ) and HG 3( ) in DTT solutions and HG 1( ), HG 2 ( ), and HG 3 ( ) in PBS solutions at 37 ^{o}C.

4. EXPERIMENTAL

4.1. Chemicals and Synthesis

Maleic anhydride was purchased from Merck. 1-(3-Dimethyşaminopropyl)-3-ethylcarbodiimide hydrochloride (98+%), 1,4-dithio-DL-threitol (98%), copper(II) bromide (98%) were purchased from Alfa Aesar Co. Tris[2-(dimethylamino)ethyl]amine (97%), 4-(dimethylamino)pyridine (>99%), copper purum p.a. (>99%), furan (>99), 3-amino-1-propanol (>99%), 4,4'-dithiodibutyric acid (95%), furfuryl methacrylate (97%), poly(ethylene glycol) methyl ether methacrylate (PEGMEMA, M_W 300), poly(ethylene glycol) methyl ether methacrylate (PEGMEMA, M_w 500) and ethyl alpha bromoisobutyrate (98%) were purchase from Sigma Aldrich. The furan-maleic anhydride adduct (**1)** and bis(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propyl) 4,4'-disulfanediyldibutanoate (**2)** were prepared according to literature procedures [44].

4.2. General Synthesis of Polymers

Monomers, initiator Cu^0 were placed in a flask and dissolved in calculated amount of DMF. Me_6TREN and $CuBr_2$ were dissolved in DMF in a different flask. Both solutions were purged with N_2 for 30 minutes. After 30 minutes, solutions were mixed and polymerization was started. After polymerization, $CuBr_2$ was eliminated by passing polymer solution through aluminum oxide. DMF was evaporated rotary evaporator. The polymer dissolved in minimum amount of dichloromethane and added to cold diethyl ether to precipitate the polymer as a solid. ^{1}H NMR ($CDCL_3$, δ, ppm), 7.44(s, 1H, CH=C*H*O), 6.39(d, 2H, furan H's), 4.93(s, 2H, COOCH_2C), 4.06(s, 2H, COOCH_2CH_2O), 3.37(s, 3H, OCH_3).

4.3. Synthesis of Maleimide Containing Crosslinker

Bifunctional crosslinker was prepared in four steps. First two steps were done similar to a previously described method to synthesize maleimide alcohol [44]. Maleimide alcohol **(**4.96 g, 22 mmol), 4,4-dithiodibutyric acid (1.14 g, 5.5 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (3.37 g, 17.6 mmol) and 4-dimethylaminopyridine (DMAP) (0.403 g, 4.65 mmol) were dissolved in dry dichloromethane (15 ml) . The solution was stirred for 24 hours at room temperature. Dichloromethane (25 mL) added to reaction mixture and diluted solution washed with saturated $NaHCO_3$. The combined organic layers were dried over anhydrous Na_2CO_3 and concentrated solution was purified by column chromatography on SiO_2 using EtOAc: CH_2Cl_2 1:2, affording **3 (**2.73 g, 80% yield). ^{1}NMR ($CDCl_3$, δ, ppm) 7.24 (s, 4H, C*H*=C*H*), 5.19 (s, 4H, C*H* bridgehead protons), 3.98 (t, 4H, OC$H_2$$CH_2$), 3.51 (t, 4H, NC$H_2$$CH_2$), 2.77 (s, 2H bridge protons), 2.67 (t, 4H, SC$H_2$$CH_2$), 2.39 (t, 4H, COC$H_2$$CH_2$), 1.97 (m, 4H, $SCH_2CH_2$$CH_2$), 1.85 (m, 4H, $NCH_2CH_2CH_2$) . Retro Diels-Alder reaction was carried out by dissolving compound **3** (2.73 g) in toluene (10 ml). The solution was refluxed at 110 ^{0}C for 24 h. The concentration solution was purified by flash chromatography on SiO_2 (EtOAc:CH_2Cl_2 1:1). 1.94 g crosslinker (4) is obtained with 90% yield. ^{1}NMR ($CDCL_3$, δ, ppm) 6.71 (s, 4H, C*H*=C*H*), 4.07 (t, 4H, OC$H_2$$CH_2$), 3.63 (t, 4H, NC$H_2$$CH_2$), 2.73 (t, 4H, SC$H_2$$CH_2$), 2.44 (t, 4H, COC$H_2$$CH_2$), 2.03 (m, 4H, $SCH_2CH_2$$CH_2$), 1.95 (m, 4H, $NCH_2CH_2CH_2$).

4.4. Preparation of the Hydrogels

Hydrogels were synthesized using three different crosslinker amounts (1.55, 2.41, 3.25, mmol mL^{-1}) at 25, 30 and 37 ^{o}C in 120 µL dioxane/water mixtures (1/4) in a standard glass vial. The mixture was sonicated for 2 minutes. Gelation took place after immersing in oil bath at different temperatures. For biomolecule loading, components of the hydrogel were mixed in biomolecule containing dioxane/water mixture. Mixture was sonicated for 10 minutes for better homogeneity of biomolecules in the solution. The samples were washed with excess amount of water before release studies to remove unloaded biomolecules.

4.5. Characterizations of Hydrogels

4.5.1 Gelation Yield of Hydrogels

Gelation yield of hydrogels were calculated gravimetrically. End point of the gelations was verified with crosslinker density. According to time sweep test in rheometer, end point of gelation process was determined. The point which elastic modulus did not increase anymore was accepted as a highest conversion of the process. After this point, hydrogels were first washed with excess amount of tetrahydrofuran then water to remove unreacted polymer and crosslinker. Afterwards, water was removed using a lyophilizer. Dried hydrogels masses (M_{dried}) was used to calculate yield of gelation processes as $(M_{dried}/M_{p+c})*100$ where M_{p+c} was the total mass of polymer and crosslinker.

4.5.2 Swelling Ratios of Hydrogels.

Equal weight dried (80 mg) hydrogels were immersed in deionized water at room temperature. The hydrogel was weighed after removal of water on the surface at periodic time points. Water uptake experiment was continued until no further increase in hydrogel weight was observed. Swelling ratios were calculated as $(M_s/M_{dried})*100$ where M_s was weight of swollen hydrogel.

4.5.3 Rheological Analysis of Hydrogels

The effect of crosslinker density and temperature on physical properties of hydrogels was investigated using Anton Paar MCR 302 rheometer. The test geometry was 15 mm diameter plate. Strain sweep test was applied to solid state hydrogel to check the linear-viscoelastic limit regime (LVR). Nonlinear strain regions represent the structural breakdown in hydrogels which were out of LVR range [45]. Frequency sweep test allows us identify the lower frequency limit that solid state hydrogel behavior can be observed

[45]. Frequency sweep test with an appropriate strain value allowed us to choose a frequency range to study on time sweep tests. Gelation process was followed with testing parameters strain value 0.5 and angular frequency value 10 rad/s. Time sweep test was run by filling gap between preheated rheometer plates with liquid state polymer-crosslinker mixture then gelation was initiated. To prevent solvent escape, closed system was used.

4.5.4 Redox Responsive Degradation of Hydrogel

Round shaped hydrogels with 2 mm thick (80 mg) was put in aqueous medium (PBS 3 mL) containing 21 x 10^{-3} M dithiothreitol. Hydrogels were incubated at 37 ^{o}C in a thermal shaker with 200 rpm. After 24 hours, solution was changed with a fresh DTT containing PBS solution. A digital camera was used to record the degradation process of bulk hydrogels.

4.6. Protein Release *in vitro*

Flourescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA) was encapsulated into hydrogels as described above. After gelation, surface of hydrogels were washed with large amount of water and water was collected. Round shaped 2 mm thick BSA included hydrogels were divided into 30 mg pieces. Hydrogels pieces were placed into empty aqueous medium (PBS, 2 ml) and DTT containing aqueous medium (PBS, 2 ml, 21x10^{-3} M) at 37 ^{o}C. The amount of protein release in the supernatant was determined with UV-vis spectroscopy.

5. CONCLUSION

Redox-responsive hydrogels were synthesized using furan-containing hydrophilic PEG-based copolymers and disulfide-containing maleimide based crosslinker. The furan-containing polymer was obtained through copolymerization of FuMA and PEGMEMA using SET-LRP. A novel disulfide containing bis-maleimide crosslinker was synthesized to install the redox-responsive linkages. Stable hydrogels are fabricated by using furan bearing polymer and bis-maleimide crosslinker through the Diels-Alder reaction in aqueous media. Three different hydrogels with various crosslink densities were fabricated and rheological analysis of hydrogels are undertaken. Degradation studies demonstrate that thus fabricated hydrogels selectively degraded in a solution containing reducing agent, and this was also confirmed by rheological methods. Different protein release profiles were obtained through the control over crosslink density and degradation.

APPENDIX

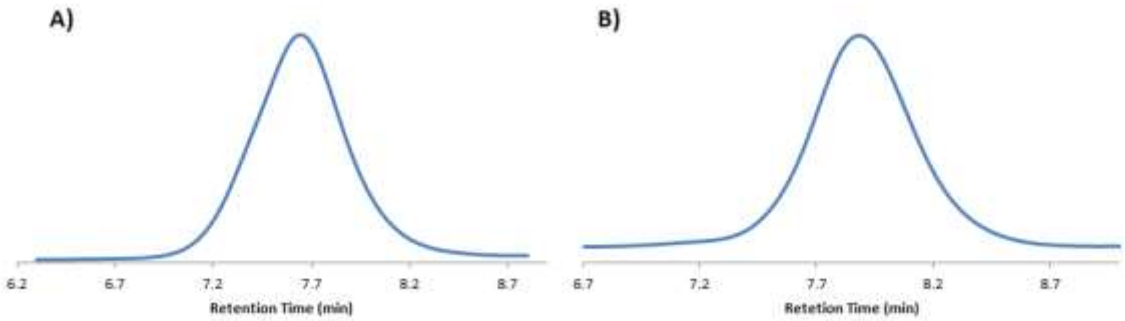

Figure A.1. A) GPC trace of polymer P-1 in THF. B) GPC trace of polymer P-2 in THF

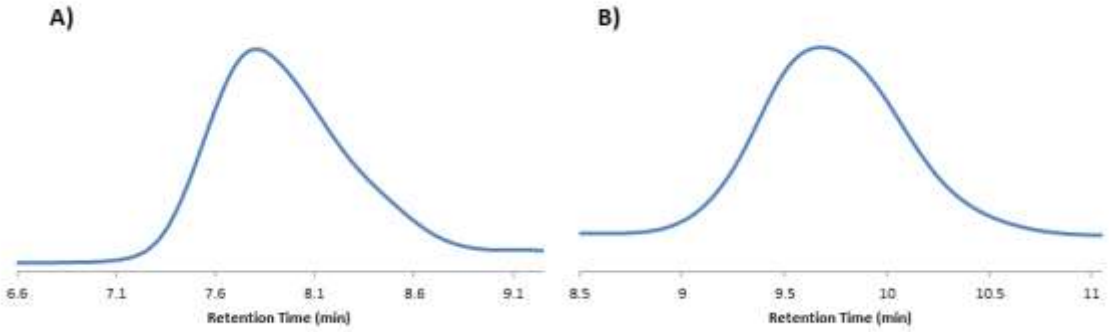

Figure A.2. A) GPC trace of polymer P-3 in THF. B) GPC trace of polymer P-4 in THF

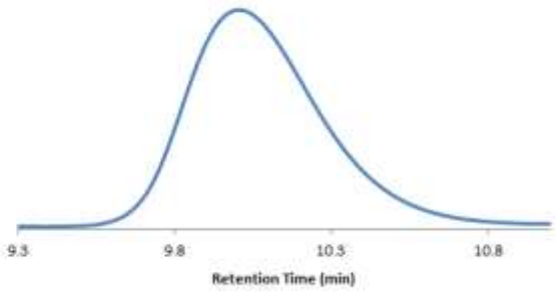

Figure A.3. GPC trace of polymer P-5 in THF

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