Thesis for the Degree of Master of Engineering

Synthesis and Characterization of Diselenide Crosslinked Polymeric Micelles via Diels-Alder Click Reaction

Diels-Alder 클릭 반응을 통한 Diselenide 교차 결합 된 고분자 미셀의 합성과 특성 규명

by

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A dissertation

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Synthesis and Characterization of Diselenide Crosslinked Polymeric Micelles via Diels-Alder Click Reaction

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Abstract

The covalent crosslinking is one of the methods to enhance the stability of micellar core. Click chemistry became a reliable strategy for covalent crosslinking due to the wide application of the polymeric modification. In this work, we reported the synthesis and characterization of stimuli and light responsive core-crosslinked micelles based on poly-(ethylene oxide)-*b*-poly (furfuryl methacrylate) (PEO-*b*-PFMA). The amphiphilic block copolymers were synthesized via single electron transfer living radical polymerization (SET-LRP). Diels-Alder click type reaction was employed to form core-crosslinked micelles using a diselenide-containing crosslinker without any catalyst. The stability of core-crosslinked micelles under the reductive-oxidative condition and near infrared exposure was investigated in this work.

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요약

공유 결합은 미셀 코어의 안정성을 향상시키는 방법 중 하나이다. 클릭 화학은 폴리머 변형에 광범위하게 적용되어 공유 가교 결합에 대한 신뢰할만한 전략이되었습니다. 이 연구에서 우리는 poly (ethylene oxide) -b-poly (furfuryl methacrylate) (PEO-b-PFMA)를 기반으로 한 자극 및 빛 반응 코어 가교 미셀의 합성과 특성을보고했다. 양친 매성 블록 공중합체는 단일 전자 이동 리빙 라디칼 중합 (SET-LRP)을 통해 합성되었다. Diels-Alder 클릭 반응을 사용하여 촉매없이 diselenide 함유 가교제를 사용하여 코어 가교 미셀을 형성하였다. 환원 - 산화 조건 및 근적외선 노출 하에서의 코어 가교 미셀의 안정성이 본 연구에서 연구되었다.

Chapter 1

General Introduction

1.1 Introduction to block copolymer

Block copolymers are macromolecules composed of sequences, or blocks, of chemically distinct repeat units. Polymerization of just two distinct monomer types leads to a class of materials referred to as AB block copolymers. Within this class, a variety of molecular architectures is possible[1]. A variety of higher order block copolymer architecture can be synthesized such as a linear diblock copolymer or linear triblock copolymer which contain two different monomeric repeating units. Multiblock copolymers can be formed by attaching triblock copolymer to one another become branched polymer include star and brush copolymer (figure 1.1).

Figure 1.1 Architecture of block copolymers; a) diblock, b) triblock, c) star, d) brush

Block copolymers have been the focus of much interest during the recent years because their constituent blocks are generally immiscible, leading to a microphase separation. Since the different blocks are linked together by covalent bonds, the microphase separation is spatially limited and results in self-assembled structures whose characteristic sizes are of the order of a few times the radius of gyration [2].

Amphiphilic block copolymers have attracted a great deal of attention in terms of their ability to form various types of nanoparticles. These polymers are obtained by the polymerization of more than one type of monomer, typically one hydrophobic and one hydrophilic, so that the resulting molecule is composed of regions that have opposite affinities for an aqueous solvent. These materials, when intended for use in drug delivery, are generally composed of biocompatible, biodegradable hydrophobic polymer blocks such as polyesters or poly(amino acids) covalently bonded to a biocompatible hydrophilic block, typically PEG. Numerous block copolymers have been synthesized, not only with a variety of block combinations but also varying hydrophilic and hydrophobic block lengths. The literature abounds with studies using amphiphilic block copolymers of different compositions and various methods of preparation that produce nanoparticles referred to as micelles, nano-spheres, core-shell nanoparticles, micelle-like nanoparticles, crew cut micelles, nanocapsules, and polymersomes[3].

1.2 Application of block copolymer in drug delivery

The recent advances in drug formulation have obviated the potential of colloidal vectors to act as efficient solubilizing agents in such cases. The capacity of block copolymer micelles to increase the solubility of hydrophobic molecules stems from their unique structural composition, which is characterized by a hydrophobic core sterically stabilized by a hydrophilic corona. The former serves as a reservoir in which the drug molecules can be incorporated by means of chemical, physical or electrostatic interactions, depending on their physicochemical properties.

Beyond solubilizing hydrophobic drugs, block copolymer micelles can also target their payload to specific tissues through either passive or active means. Prolonged in vivo circulation

times and adequate retention of the drug within the carrier are prerequisites to successful drug targeting. Long circulation times ensue from the steric hindrance awarded by the presence of a hydrophilic shell and the small scale (10–100 nm) of polymeric micelles. Indeed, micelles are sufficiently large to avoid renal excretion (>50 kDa), yet small enough (<200 nm) to bypass filtration by inter-endothelial cell slits in the spleen. Drug retention, in turn, is dependent on micelle stability and polymer-drug interactions. Many approaches are being employed to enhance the physical stability of the carrier, improve its resistance towards dissociation upon entering the bloodstream, and tailor its properties to better suit those of the incorporated drug[4].

1.3 Block copolymer micelles

Whenever amphiphilic block copolymer chains are dissolved at a fixed temperature and in a selective solvent for one of the blocks, they self-associate through a closed association process to form micelles similarly to low-MW surfactants. The critical concentration at which the first micelle forms is called the critical micelle concentration, or CMC. As the concentration of block copolymer chains increases in the solution, more micelles are formed while the concentration of non-associated chains, called unimers, remains constant and is equal to the value of the CMC. This ideal situation corresponds to a system at thermodynamic equilibrium[5].

Due to the unique structure of amphiphilic molecules, they have a tendency to accumulate at the boundary of two phases and thus are termed surfactants. In aqueous solutions, amphiphilic molecules orientate themselves so that the hydrophobic blocks are removed from the aqueous environment in order to achieve a state of minimum free energy. As the concentration of amphiphile in solution is increased, the free energy of the system begins to rise due to unfavorable interactions between water molecules and the hydrophobic region of the

amphiphile resulting in the structuring of the surrounding water and a subsequent decrease in entropy.

At a specific and narrow concentration range of amphiphile in solution, termed the critical micelle concentration (CMC), several amphiphiles will self-assemble into colloidal-sized particles termed micelles. The formation of micelles effectively removes the hydrophobic portion of the amphiphile from solution minimizing unfavorable interactions between the surrounding water molecules and the hydrophobic groups of the amphiphile. If the amphiphile concentration in solution remains above the CMC, micelles are thermodynamically stabilized against disassembly. Upon dilution below the CMC, micelles will disassemble with the rate of disassembly being largely dependent on the structure of the amphiphiles and interactions between the chains[3–5].

1.4 Crosslinked micelles

A major disadvantage of micelles is their dynamic nature which leads to instabilities at high temperature, at low concentrations and under certain changes in solvent conditions. As a result, there has been significant interest in the stabilization of micelles and in particular, polymer micelles. In nature, stabilization of self-assembled structures is often achieved by covalent cross-links and, by analogy, it has been demonstrated that the selective formation of such cross-links between specific blocks within a copolymer micelle is possible and affords a single, nanostructured macromolecule or nanoparticle. Thus, the formation of cross-links throughout a specific domain offers stability to the nanostructured assemblies by providing reinforcement to the weak intermolecular interactions that facilitate polymer micelle assembly and existence.

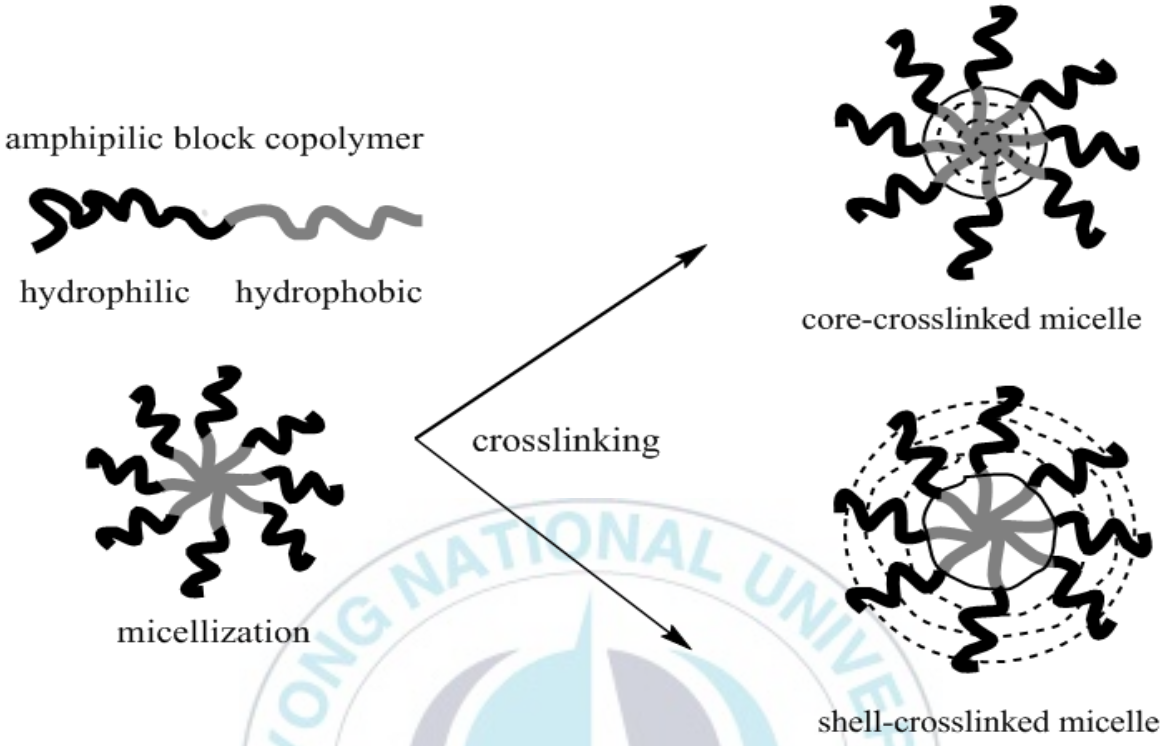


Figure 1.2 Representation of formation of shell-crosslinked or core-crosslinked micelles

There are several potential locations for crosslinking within diblock polymer micelles, including at the core chain end, within the core domain, at the core-shell interface, throughout the shell layer, and on the surface (Figure 1.2). The location of this cross-linked domain can dramatically affect the physical and chemical properties of the resulting materials [6].

1.5 Introduction of radical polymerization

Reversible-deactivation radical polymerization (RDRP) methods have been developed over the past two decades, which allow polymers to be synthesized with control over the molecular weight and architecture comparable to ionic polymerizations, although with tolerance to

functional groups and impurities similar to conventional radical polymerizations. These RDRP methods reversibly cap active radicals to dormant chains, thereby gaining control over the macromolecular structure. The three most popular RDRP methods are nitroxide mediated polymerization (NMP) reversible addition–fragmentation chains transfer (RAFT) polymerization, and atom transfer radical polymerization (ATRP). One of the most versatile and mild ways of controlling the structure of the macromolecule is to use transition metal species to mediate the polymerization. In ATRP, transition metal species catalytically activate alkyl halides to generate radicals and deactivate the radicals back to alkyl halide species after several monomers have been added. In classical ATRP the low oxidation state metal complex, for instance, Cu^{I}, is used to activate an alkyl halide generating a radical and a Cu^{II} halide complex. In this ATRP system, the Cu^{II} halide complex will deactivate the radical, reforming the Cu^{I} complex and the alkyl halide[7].

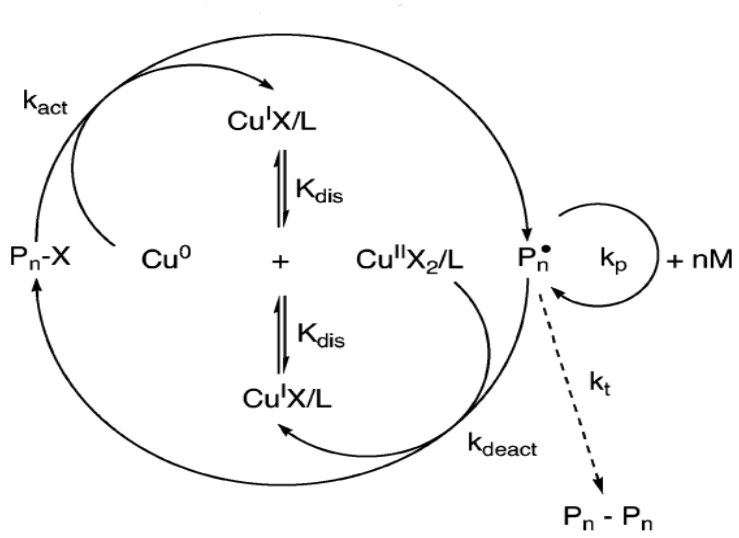
Since ATRP obeys the persistent radical effect (PRE), traditionally, catalyst concentrations greater than 1000 parts per million (ppm) were needed to maintain a high polymerization rate. In the past few years, techniques have been developed that give well-controlled polymerizations, using less than 100 ppm of the catalyst. This is achieved by continuously regenerating the activator complex, by reducing the deactivator, or compensating for the loss of activator species through supplemental activation reactions. These processes include activators regenerated by electron transfer (ARGET) ATRP, and initiators for continuous activator regeneration (ICAR) ATRP, electrochemically mediated ATRP, and photochemically mediated ATRP[7].

An interesting scenario occurs when zerovalent metals are introduced into the system since a wide variety of reactions are possible due to the different oxidation states of the metals. Cu^{0}

was first used in RDRP in 1997, and the method has become popular in controlled polymer synthesis since 2006 when Percec and coworkers published a paper on efficient RDRP in the presence of Cu^0. Since the development of RDRP in the presence of Cu^0, various well-defined complex polymers, such as hyperbranched, decablock copolymers, pentablock star polymers, and dendritic structures have been prepared using Cu0. There are several advantages of using Cu^0 in a polymerization, including low concentrations of the soluble Cu species, simple removal and reuse of unreacted solid Cu^0 and control of the polymerization rate by the amount of ligand and the surface area of Cu^0. Nevertheless, it is important to characterize the reaction mechanism and determine the effect of different reaction conditions on the resulting polymer to optimize the reaction conditions for targeted polymer structures[8].

1.6 Single electron transfer living radical polymerization (SET-LRP)

Since the last years of the 20th century, a number of controlled or living radical polymerization (LRP) techniques have been reported. Single Electron Transfer (SET)-LRP was introduced by Percec et al. [9]. The authors claim that SET-LRP is catalyzed by extremely reactive Cu(0) that is formed by low activation energy outer-sphere single-electron-transfer. The reaction is controlled or deactivated by Cu(II) species that are formed via the same process (Figure 1.3). It has been reported that SET-LRP is very effective at room temperature and that extremely high molecular weights can be obtained in conjunction with a low PDI. Even in the presence of typical radical inhibitors such as phenol, SET- LRP shows control over the molecular weight distribution and exhibits a high reaction rate. The mechanism of SET-LRP is still under debate. Matyjaszewski and coworkers [10] reported that according to their results the reaction follows the same mechanism as ARGET-ATRP and the role of Cu(0) is limited to that of the reducing agent[11].



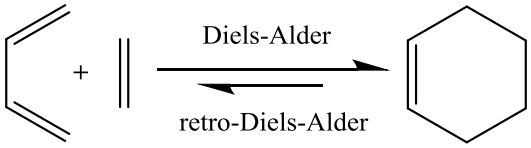
Scheme 1.1 The SET-LRP mechanism as proposed by Percec et al.[9].

SET-LRP proceeds at 25 °C in H_2O and uses Cu(0) and/or "nascent" Cu(0) generated in situ by the disproportionation of various Cu(I) precursors as a catalyst. In this polymerization, Cu(0) species act as electron donors, and the initiator and dormant propagating species act as electron acceptors. The Cu(I) species generated during the formation of radicals spontaneously disproportionate into extremely reactive nascent Cu(II) and Cu(0) atomic species that mediate the initiation and the reversible termination. This disproportionation generates, by a self-regulated mechanism, in situ, the Cu(II) species. By this mechanism, the inactive Cu(I) species are spontaneously consumed and the catalytically active Cu(0) species are continuously produced. This polymerization process takes place in H_2O, protic, dipolar aprotic, and other polar solvents that, in the presence of N-ligand was discovered to disproportionate Cu(I) into Cu(0) and Cu(II). These solvent and ligand combinations also favor a SET process. SET-LRP occurs under very mild reaction conditions, at room temperature and below, use a catalytic rather than a stoichiometric amount of catalyst, and although proceeds ultrafast, generates

polymers with unprecedently high molecular weight. SET-LRP is general and applies to both nonactivated and activated monomers containing electron-withdrawing functional groups, such as vinyl chloride and other halogenated monomers, acrylates, and methacrylates. It also applies to organic reactions and tolerates a diversity of functional groups[7,10]

1.7 Diels-Alder click chemistry

The potential of "click chemistry" for materials synthesis has been increasingly recognized and has already resulted in the development of a wide range of interesting materials. Because of their high selectivity, high yields, and exceptional tolerance toward a wide range of functional groups, and reaction conditions click reactions have recently attracted increased attention in polymer synthesis as well as polymer modification. Diels-Alder (DA) click reactions have played an essential role in various aspects of polymer synthesis and chemical modification of the polymers. The use of polymeric systems based on acrylic derivatives as biomaterials for clinical applications has increased because of their excellent biocompatibility and long-term stability. Nowadays, there is a considerable interest in the synthesis of new types of polymeric materials and also in the modification of existing polymers in order to alter their properties to meet requirements for new applications. Recently, the DA reaction based on macromolecular chemistry has attracted much attention, particularly for providing new materials[13].



Scheme 1.2 General mechanism of Diels–Alder/retro Diels–Alder reaction of dienophile and diene as proposed by Tasdelen [14]

This click reaction has been perfectly adapted for polymer synthesis and has often been considered for the preparation of complex architectures such as dendrimers, stars and graft polymers due to its simplicity, versatility, and efficiency. Likewise, the combination of the Diels-Alder reaction with living/controlled polymerization methods is the preferential route for synthesizing such a variety of functional polymers and architectures. However, what can be said is the most attractive aspect of the Diels-Alder reaction is its thermal reversibility, which is done through the retro-DA reaction thus enabling its use in a variety of applications. Hence, the decoupling reaction, as well as the adduct formation, can occur at a range of different temperatures depending on the diene/dienophile combination[15].

1.8 Aim and outline

The aim of this thesis is to give a facile approach of core-crosslinked polymeric micelles prepared by SET-LRP technique. The studies carried out in this thesis are divided into three parts. The first part is the synthesis of the new crosslinker containing selenide and bismaleimide for a clickable agent for crosslinking reaction. The second part is the preparation of PEO-PFMA block copolymers with furan functional group as an active site for Diels-Alder crosslinking click reaction. The third part of this thesis is the responsive investigation of core-crosslinked micelles under reductive and oxidative conditions also under exposure to near infrared light source.

1.9 References

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Chapter 2

Synthesis of Diselenide containing crosslinker for Diels Alder (DA) Click Reaction

Click chemistry is found in nearly all areas of modern chemistry from polymer-drug discovery to materials science and also one of the promising ways for crosslinking polymer modification. The premier examples of click chemistry are cycloaddition reactions (1,3-dipolar family also Diels-Alder), nucleophilic ring opening reactions, carbonyl chemistry of the non-aldol type and addition to carbon-carbon multiple bonds. The purpose of this research is utilizing Diels-Alder click chemistry strategy for crosslinking polymers with synthesized diselenide containing crosslinker. Diselenide was chosen due to stimuli-sensitive and reductive biodegradable ability. Kinds of click-able diselenide crosslinker containing maleimide functional group were prepared by esterification of 3,3-diselaneidyldipropionic acid with hydroxyethyl maleimide. Bis-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)3,3'-diselanediyldipropionate crosslinker was prepared for Diels-Alder click reaction. ^{1}H and ^{13}C NMR were used to characterize selenium containing crosslinker.

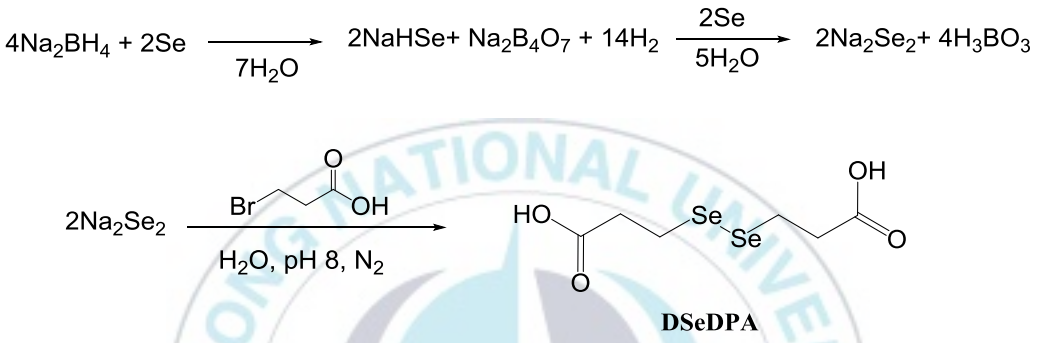
2.1. Introduction

Organic selenium compounds have demonstrated to be excellent candidates for stimuli-responsive materials due to their dual respond to both oxidants and reductants. Compared with other redox-responsive materials, such as polysulfides, the redox responsiveness of selenium-containing polymers may be more sensitive and faster. Considering that inflammatory cells often exhibit a more oxidative atmosphere intra-cellularly than the healthy ones, it is significant to develop oxidation-responsive drug vehicles which can disassemble and release the loaded drugs in an oxidative media[1].

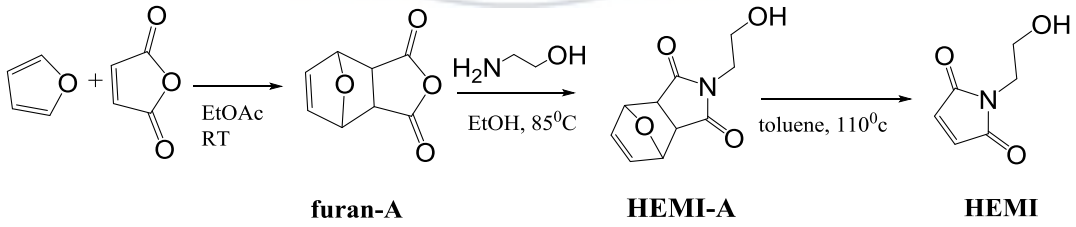
Selenium also possesses unique chemical properties owing to its special electronegativity and atomic radius. The radius of the selenium atom is bigger than that of sulfur, and the electronegativity of selenium is weaker than that of sulfur and makes it easier for low valence state selenium compounds to be oxidized[2–4]. Diselenide is a promising candidate for a dual redox response due to its good activity in the presence of either oxidants or reductants. Normally, diselenide bonds can be oxidized to selenic acid in the presence of oxidants and reduced to selenol in a reducing environment[4,5]. The previous study also reported diselenide bond can be cleaved in the presence of reactive oxygen species (ROS) and the red light (range from 600 to 780 nm) triggered disassembly of diselenide-containing micelles with the utilization of porphyrin derivatives that can generate singlet oxygen under red light[6,7].

Herein we attempt to take advantage of the unique stimuli-responsive characteristic from diselenide bond and introduce Diels-Alder (DA) click chemistry into a dual responsive crosslinker. The diselenide bond attached to the dicarboxylic acid form then reacted with hydroxyethyl maleimide forming diselenide ester crosslinker containing maleimide functional group. This new crosslinker is a click-able compound that can be utilized via Diels-Alder reaction. The diselenide

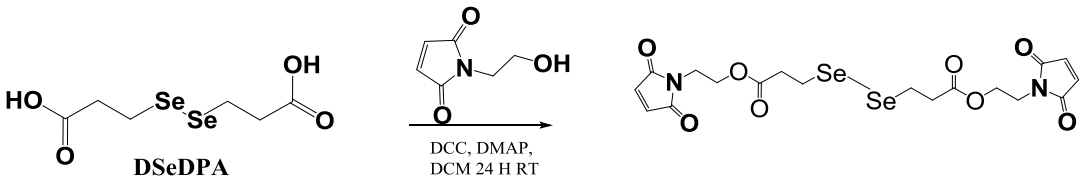
crosslinker was designed to the further step by attaching this crosslinker into furan bearing chain polymer would make crosslinking reaction which can be used as drug delivery vehicles in a controlled manner. The excellent redox-responsive formation of core crosslinked polymeric micelles using DA rection of amphiphilic diblock copolymer PEO-PFMA with disulfide maleimide containing crosslinker as drug delivery vehicles for doxorubicin has been reported in the previous work[8].



Scheme 2.1 Synthetic route for 3,3'-diselanediyldipropionic acid proposed by Cheng[9]



Scheme 2.2 Synthetic route for hydroxyethyl maleimide (HEMI) proposed by Gramlich[10].



Scheme 2.3 Synthetic route for diselenide bismaleimide containing crosslinker(Bis(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)3,3'-diselanediyldipropionate)

Synthesis of diselenide bismaleimide containing crosslinker was performed through three steps. The first step was synthesized 3,3'-diselanediyldipropionic acid (DSeDPA) by reacting sodium borohydride with selenium in water. The second step was preparing the alcohol-containing maleimide by reacting furan with maleic anhydride then functionalized with ethanolamine to form hydroxyethyl maleimide (HEMI). The third step was the esterification of DSeDPA with HEMI using DCC coupling agent, after purification by flash column chromatography the crosslinker was characterized by ^{1}H NMR.

2.2. Experimental Section

2.2.1. Materials

Selenium powder 99.99%, sodium borohydride (NaBH4) 99%, 3-bromopropinoic acid 97%, 4-(dimethylamino)pyridine (DMAP) 99%, *N*,*N*′-Dicyclohexylcarbodiimide (DCC) 99%, furan 99%, maleic anhydride 99%, ethanol 98% were supplied by Aldrich. Ethanolamine 99% was purchased from Fluka. Sodium carbonate(Na_2CO_3) was purchased from Duksan Pure Chemical Dichloromethane, ethyl acetate, and toluene solvents were HPLC grade purchased from Duksan Pure Chemical.

2.2.2. Synthesized of 3,3'-diselanediyldipropionic acid (DSeDPA)

3,3'-diselanediyldipropionic acid (DSeDPA) was synthesized according to the previous work[9,11]. Selenium powder (4.73 g, 60 mmol) in 20 mL of deionized water was added to a two-necked flask under a nitrogen atmosphere. $NaBH_4$ (4.54 g, 120 mmol) in 50 mL cold deionized water was added dropwise to selenium suspension. The mixture was stirred at 0°C until getting a colorless solution which indicated complete dissolution of selenium. Another quantity of selenium (4.73 g, 60 mmol) was added 30 minutes after the colorless solution is formed. The mixture then was heated until 105°C for 30 minutes until brown mixture is formed. 3-bromopropionic acid (18.36 g, 120 mmol) was dissolved in 30 mL deionized water and pH was adjusted until 8 with sodium carbonate. The basic solution of 3-bromopropionic acid was added to the brown solution and stirred under nitrogen atmosphere at room temperature for 12 hours. Subsequently, the mixture was exposed to the air for 2 hours and was filtered. The yellow filtrate then acidifies by 1M HCl solution until pH 3 and extracted twice with ethyl acetate. The organic layer then washed with deionized water, dried over anhydrous magnesium sulfate, filtered and recrystallized twice in ethyl acetate. The light yellow powder then was dried under vacuum and obtained 11.3 g (a 62% yield). ^{1}H-NMR. (400MHz, d_6-DMSO) :δ2.71 (t,2H), δ3.05(t,2H). ^{1}H NMR spectra were recorded on JNM-ECP 600 (JEOL).

2.2.3. General procedure to synthesized HEMI

2.2.3.1. Synthesized of 4,10-Dioxatricyclo[5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione (Furan A)

Maleic anhydride (13.28 g, 0.135 mol), furan (12.3 g, 0.18 mol) and ethyl acetate 50 mL were added to round bottom flask and stirred at room temperature for 24 hours. The mixture then was filtered, washed by diethyl ether, and dried under **vacuum**. The product (furan-A) was used without further purification (yield 80%). ^{1}H-NMR (600MHz, d_6-DMSO) δ: 6.58 ppm (s, 2H, –

CHCH=CHCH–), 5.35 ppm (s, 2H, –CHCH=CHCH–), 3.31 ppm (s, 2H, O=CCH). ^{1}H NMR spectra were recorded on JNM-ECP 600 (JEOL).

2.2.3.2. Synthesized of 4-(2-Hydroxyethyl)-10-oxa-4-aza-tricyclo[5.2.1.02,6]- dec-8-ene-3,5-dione (HEMI A)

Furan-A (9 g, 0.054 mol) EtOH (15 mL) were added and mixed to a round bottom flask with. A solution of MEA (3.38g, 0.056 mol) and EtOH (15 mL) was added dropwise to the Furan-A solution. The mixture was refluxed at 85 °C after 6 hours the mixture turned an orange solution. After the reaction, the solution was cooled 24 hours and the crystallized product was removed via suction filtration. The collected crystals were dried under vacuum at room temperature. The colorless product (HEMI-A) was used without further purification (yield 50%).^{1}H-NMR (600 MHz, DMSO-d6) δ: 6.55 (s, 2H, – CHCH=CHCH–), 5.12 (s, 2H, –CHCH=CHCH–), 4.77 (board, 1H, NCH_2CH_2OH), 3.41 (m, 4H, NCH_2CH2OH), 2.92 (s, 2H, O=CCH). ^{1}H NMR spectra were recorded on JNM-ECP 600 (JEOL).

2.2.3.3. Synthesized of 1-(2-Hydroxyethyl)-1H-pyrrole-2,5-dione (HEMI)

HEMI-A (5.6 g) and toluene (50 mL) were added to a 3-neck round bottom flask with a stir bar. The reactor was continuously purged with nitrogen while the solution was refluxed at 110 °C for 5 hrs. Upon cooling at 0 °C for 2 hours, a white solid (HEMI) was collected by suction filtration and washed with petroleum ether (yield 90%).^{1}H NMR (600 MHz, DMSO-d6) δ: 7.01 (s, O=CCH=CHC=O), 4.79 (s, 1H, NCH_2CH_2OH), 3.45 (m, 4H, NCH_2CH_2OH). ^{1}H NMR spectra were recorded on JNM-ECP 600 (JEOL).

2.2.4. Synthesis of diselenide containing crosslinker for DA reaction

HEMI (5.00 g, 0.035 mol) DCC (8.04 g, 0.039 mol) and DMAP (47.61 mg, 0.0039 mol) were dissolved in 10 mL dry dichloromethane and cooled for 30 minutes in an ice bath. DSeDPA (5.17 g, 0.017 mol) in 5 mL dichloromethane was added dropwise into HEMI mixture and continue stirring in an ice bath for another 30 minutes, the reaction was then brought to room temperature and stirred further for 24 hours.the white precipitated dicyclohexylurea by-product was filtered off and the product solution was concentrated. The product solution was washed with water, dried over MgSO4, and then concentrated in a rotary evaporator. The product was finally purified by flash column chromatography n SiO_2 (EtOAc: $CHCl_2$) affording 2.52 g (75% yield).^{1}H-NMR (600 MHz, $CDCl_3$) δ: 6.73 (s, 4H, CH=CH), 4.25 (t, 4H, OCH_2) 3.80 (t, 4H, NCH_2), 3.04(t, 4H, OCH_2CH_2), 2.77(t 4H, CH_2CH_2Se). ^{13}C-NMR (600 MHz, $CDCl_3$) 171.51, 170.33, 133.88, 61.12, 36.87, 35.96, 22.07.^{1}H and ^{13}C NMR spectra were recorded on JNM-ECP 600 (JEOL).

2.3. Result and Discussion

2.3.1. Synthesis of 3,3'-diselanediyldipropionic acid (DSeDPA)

Inspired by excellent disulfide bonds for linker agent in drug and gene delivery, diselenide bonds have been considered as a potential candidate for stimuli-sensitive design for drug and gene delivery due to their characteristic[9]. In order to achieve crosslinking of diselenide bonds with DA click strategy, modification of functional group was a crucial step. In this work diselenide bond was introduced to carboxylic acid form, by reacting sodium diselenide (Na_2Se_2) with 3-bromopropionic acid. Sodium diselenide was produced by reacting selenium powder with sodium borohydride in a protic solvent. This kind of reaction was rapidly and exothermically at room temperature[11]. The Na_2Se_2 moiety was depending on selenium and sodium borohydride ratio.

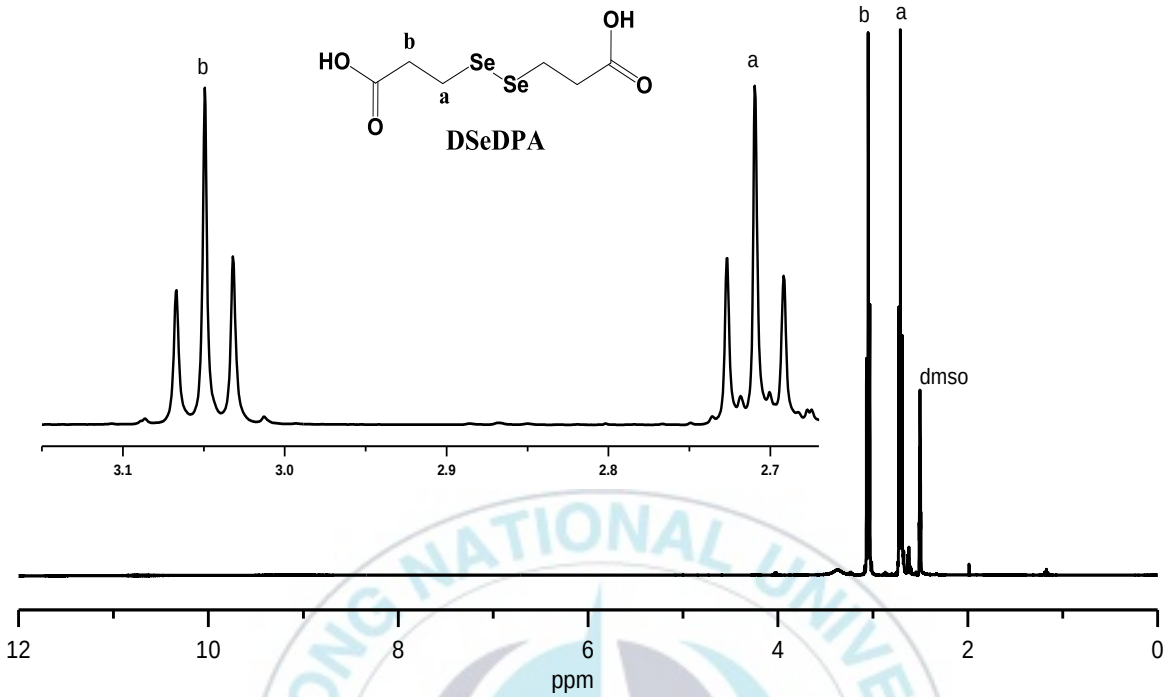


Figure 2.1 ^{1}H-NMR spectrum of 3,3'-diselanediyldipropionic acid

When the ratio of borohydride to selenium 2:1 sodium hydrogen selenide (NaHSe) colorless aqueous solution was produced. When the ratio 1:1 Na_2Se_2 was produced as brown aqueous. 3-bromopropionic acid was added to sodium diselenide aqueous to obtain the yellow desired product. After 12 hours, unreacted Na_2Se_2 was oxidized by exposure to the atmosphere. The Purification steps had acidifed the supernatant to remove unreacted acid, then extraction and recrystallization with ethyl acetate. The product was a fine yellow light powder with 62% yield. This product then characterized by ^{1}H-NMR. The presence of methylene groups (-OCH_2-) resonance in the 1H NMR spectra near 3.05 ppm and methylene groups(-CH_2Se-) near 2.71 ppm confirmed the structure of DSeDPA.

2.3.2. General procedure to synthesized HEMI

The HEMI compound was synthesized through three steps. The first step began with the reaction between furan and maleic anhydride to produce a protected group of furan-A. The second step was insertion amine group to produce protected maleimide form HEMI-A.The last step was deprotected maleimide form through retro Diels-Alder by refluxing HEMI-A with toluene in a nitrogen atmosphere.

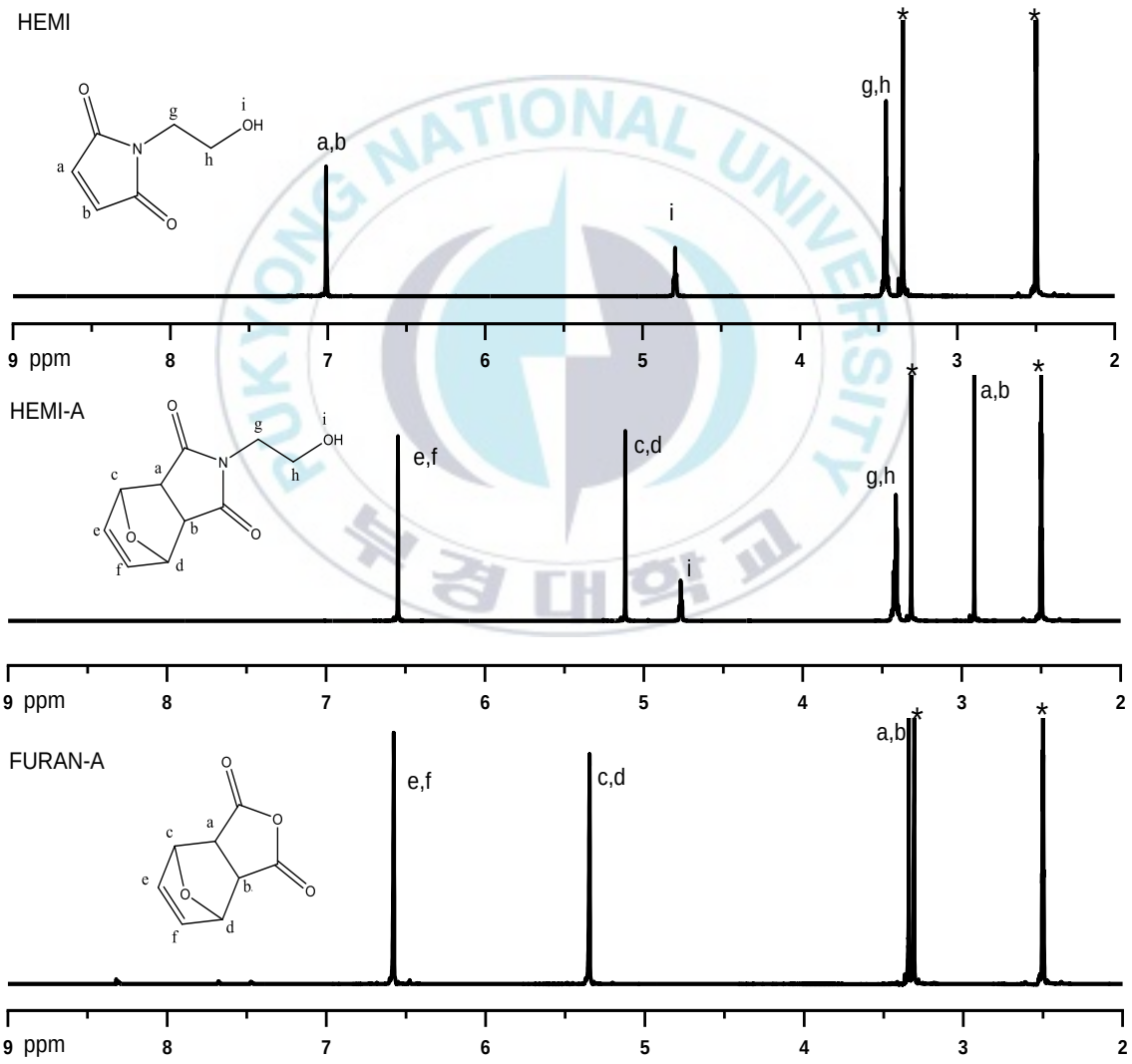


Figure 2.2 ^{1}H-NMR spectrum of furan-A, HEMI-A, and HEMI

The composition and purity of the furan-A, HEMI-A, and HEMI were deduced from ^{1}H NMR spectrum as shown in figure 2.2. The proton resonance of CH-CH (a,b) from furan-A 3.31 ppm was shifted to 2.92 ppm after insertion of MEA, then new peak at 7.01 ppm appeared from HEMI indicated that deprotection reaction occurred. The Insertion of MEA also indicated by the appearance of a new peak of OH (i) at 4.79 ppm and multiplet peak CH_2-CH_2 (g,h) from MEA around 3.4 ppm. The disappearance of HC=CH around 6.5 ppm strongly indicated that unmasking HEMI-A became HEMI was successful.

2.3.3. Synthesis of diselenide containing crosslinker for DA reaction

The click-able crosslinker containing diselenide was synthesized through esterification of DSeDPA (3,3'-diselanediyldipropionic acid) with hydroxyethyl maleimide (HEMI) in presence of DCC coupling agent and DMAP as a catalyst.

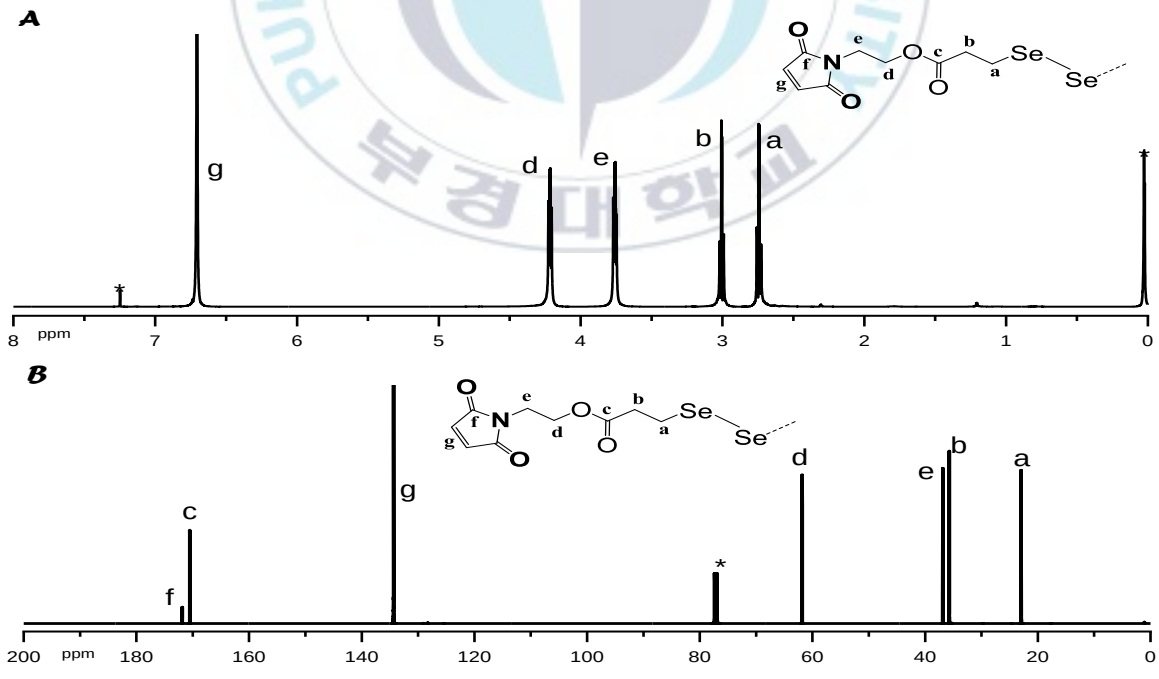


Figure 2.3 A ^{1}H-NMR and B ^{13}C-NMR spectrum of diselenide containing crosslinker

The critical steps were confirmed by ^{1}H-NMR and ^{13}C NMR. The triplet proton resonance peak at 3.04 ppm and 4.25 ppm indicated the presence of ester bond. The Peak of ester bond also shown in ^{13} C-NMR spectra at 170.33 ppm. Insertion of hydroxyl ethyl maleimide has been proofed by chemical shift of CH2-CH2. Before esterification CH2-CH2 from HEMI appeared as a multiplet at 3.45 ppm, after esterification it was shifted into triplet at 3.8ppm and triplet at 4.25 ppm.

2.4. Conclusion

The new crosslinker containing diselenide bond and maleimide functional group for Diels-Alder reaction was successfully synthesized by esterification DSeDPA and HEMI. DSeDPA was synthesized by reacting 3-bromopropinoic acid with sodium diselenide as a precursor. HEMI was prepared through three steps which are synthesized masking furan then reacting with MEA to produce HEMI-A, the last step was unmasking HEMI-A to produce HEMI. The new crosslinker then characterized by ^{1}H-NMR and ^{13}C-NMR. Diselenide crosslinker with maleimide functional group would be a promising material for modification polymer through click chemistry strategy due to responsive characteristic from selenium and versatile properties from maleimide

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Chapter 3

Synthesis and characterization of diselenide crosslinked polymeric micelles via Diels-Alder click reaction

A well-defined amphiphilic block copolymer, poly (ethylene oxide)-b-(poly (furfuryl methacrylate) (PEO-b-PFMA) was prepared by single electron transfer living radical polymerization (SET-LRP) using tris(dimethylamino)ethylamine (Me6TREN) as a ligand. The block copolymer formed sub-100 nm micelles in water with PEO as a shell and PFMA as a core. Diels-Alder click type reaction was employed to form core-crosslinked micelles using a diselenide-containing crosslinker without any catalyst. The block copolymer and micelles were characterized by gel permeation chromatography, nuclear magnetic resonance, Fourier transformation infrared spectroscopy, dynamic light scattering analysis, and transmission electron microscopy. The stability of core-crosslinked micelles under irradiation of near infrared was also investigated. The diselenide crosslinked micelles decomposed under the irradiation of red light (780nm) in the presence of reactive oxygen species from indocyanine green. The responsive near-infrared light core-crosslinked micelles can be a promising carrier for drug delivery applications.

3.1 Introduction

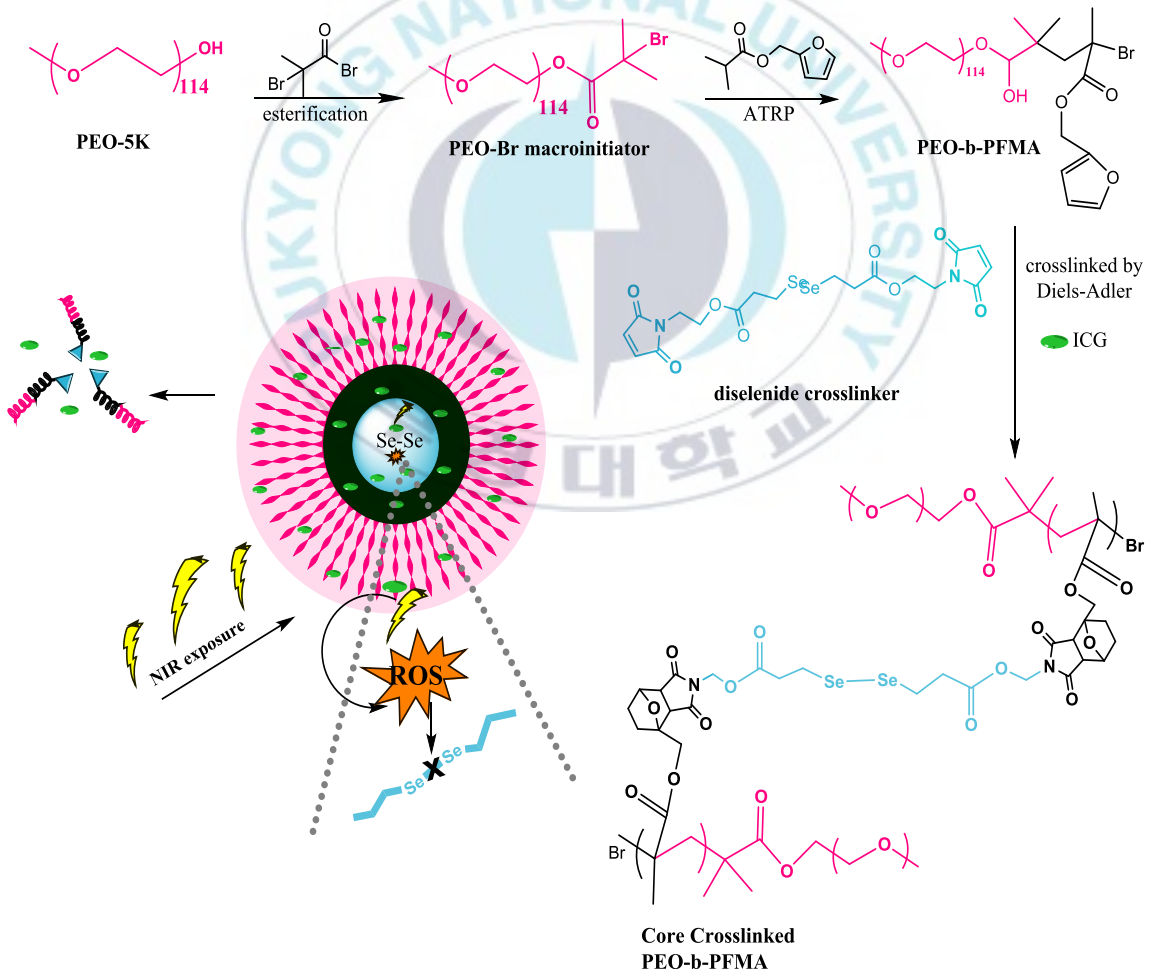
In recent years, crosslinked polymeric materials have drawn interest in pharmaceutical research due to their efficient ability in drug delivery system[1]. Manifold kinds of crosslinker have been developed including acetal, hydrazine bond, ester, and disulfide. Among them, disulfide has been widely introduced for reductive drug vehicle design[2]. Similar properties with disulfide bonds, diselenide bonds also have been considering as potential stimuli-responsive design. As reported recently, diselenide bonds possess a reductive biodegradable ability similar to that of disulfide bonds[3]. The diselenide bond was designed to have responsive in the reduction-oxidation environment, and in particular, it can be cleaved in the presence of reactive oxygen species (ROS)[4].

Xu and co-workers reported that the red light (range from 600 to 780 nm) triggered disassembly of diselenide-containing micelles with the utilization of porphyrin derivatives that can generate singlet oxygen under red light[5]. Indocyanine green (ICG) is an organic dye that can produce of ROS (such as singlet oxygen, superoxide anions, and hydroxyl radicals) upon NIR light irradiation. Tian group was reported that introducing diselenide bond into a polymer network with the incorporation of ICG under NIR light could be used to trigger the disassembly polymer network due to cleaved of diselenide bond[6]. This dye has been approved by the US Food and Drug Administration (FDA) for human medical imaging and diagnosis[7]

Among the various “click chemistry” reactions, Diels-Alder (DA) cycloaddition has been employed widely in the crosslinking polymeric system[8]. Diels-Alder reaction has great potential due to its advantageous characteristic including no metal catalyst, simple reaction condition, simple product isolation and has a special feature, DA reaction, and retro-DA are thermal reversibility and the decomposition can be controlled by temperature[9]. The modification of block copolymer would be a suitable strategy to show DA reaction allow facile

access to polymeric architecture. For example, DA"click" reaction, [4 + 2] system, based on the coupling of furan protected maleimide- and anthracene-end functionalized polymers have been successfully used to generate numerous block copolymers[10].

The Preparation of crosslinking polymer utilizing DA reaction of the amphiphilic block have been reported by Le and coworkers, redox-responsive core crosslinked micelles from poly(ethylene oxide)-b-poly(furfuryl methacrylate) was crosslinked by DA strategy with disulfide crosslinker give a good result for drug delivery system. De-crosslinking reaction to release drug occurred in the reductive-oxidative environment and certain pH circumstance due to the disulfide bond[11].



Scheme 3.1 Preparation of core crosslinked of PEO-PFMA block copolymer

Herein in this work, the dual responsive core-crosslinked polymer from poly(ethylene-oxide)-b-poly(furfuryl-methacrylate) PEO-*b*-PFMA utilizing DA reaction was proposed. The PEO-*b*-PFMA diblock copolymers were prepared by single electron transfer living radical polymerization (SET-LRP). Then diselenide containing crosslinker with maleimide functional group was introduced into furfuryl methacrylate polymeric network for the core-crosslinked polymeric system. The stability of the core-crosslinked polymeric system was investigated in the reductive-oxidative environment and under the irradiation of infrared light with the presence of ICG.

3.2 Experimental Section

3.2.1 Materials

Poly (ethylene glycol) methyl ether (average Mn 5000), trimethylamine, furfuryl methacrylate (97%), furan (99%), N, N′-Dicyclohexylcarbodiimide (DCC), and 1,4-dithiothreitol (DTT) was purchased from Sigma-Aldrich. Copper wire (0.5 mm), 2–bromoisobutyryl bromide (97%), and dimethylamino pyridine (99%) were purchased from Alfa Aesar. Dimethylformamide, dichloromethane, acetonitrile, ethyl acetate, and tetrahydrofuran were purchased from Duksan Pure Chemical Co. Ltd. Tris (dimethylamino) ethylamine (Me6TREN) was prepared according to the previous literature12. Diselenide containing bismaleimide crosslinker was synthesized in the same manner as in the previous studies.

3.2.2 Preparation of PEO-Br macroinitiator

The PEO-Br macroinitiator was prepared according to the previous study 13. 0.348 g (3.44 mmol) of trimethylamine and 0.6303 g (5.16 mmol) of DMAP in 14 mL of dry dichloromethane were placed in a round-bottom three-neck flask. The mixture was cooled to 0OC, after 30 minutes 1.98 g (8.6 mmol) of 2-bromoisobutyryl bromide in 14 mL

dichloromethane was added to the mixture to form a light yellow dispersion. PEO-5k (17 g, 3.44 mmol) in 70 mL of dichloromethane was added dropwise to nitrogen atmosphere during 1 hour. Subsequently, the temperature was allowed to rise to room temperature. The reaction was continuously stirred for 18 h. The solution was then filtered, concentrated and precipitated in cold diethyl ether. The product was recrystallized in ethanol and washed with cold diethyl ether and dried under vacuum. 15 g of the PEO-Br macro-initiator was obtained with 94% yield. 1H NMR (600 MHz, CDCl3) δ 4.29 (t, 1.94H), 3.67 – 3.51 (m, 467H), 3.34 (s, 3.09H), 1.90 (s, 6H).

3.2.3 Synthesis PEO-b-PFMA via SET-LRP

In typical procedure the ratio of reactants used were [monomers] : [macro-initiator] : [Cu-wire] : [Me6TREN] ; 30 : 1 : 8 : 0.36 (molar equivalents) . Furfuryl methacrylate (1.881 g, 11.320 mmol) PEO5K-Br (2 g, 0.377 mmol), Cu wire (12.5 cm, d= 0.5 mm, 0.205 g) Me6TREN (0.0312 g, 0.1258 mmol) and the solvent (DMF, 6 mL) were added into a round bottom flask. The solution was purged with N2, and the reaction proceeded for 18 h at room temperature. To remove copper from the solution, the resulting mixture was passed over neutral alumina column, with dichloromethane as an eluent. The product was concentrated on a rotary evaporator and precipitated in cold diethyl ether. The final product was collected and dried under vacuum at room temperature for 24 h (2.91 g, yield 75%). 1H NMR (600 MHz, DMSO-d6) δ 7.63 (s, 1H), 6.45 (d, 2H), 4.88 (s, 2H), 1.87 – 1.41 (m, 2H), 0.77 – 0.20 (m, 3H).

3.2.4 Core-crosslinked of PEO-b-PFMA by Diels- Alder click chemistry

Crosslinking reaction of PEO-b-PFMA functional copolymer was carried out by dissolving the copolymer (50 mg, 0.013 mmol of furfuryl moieties) and the selenium-containing crosslinker (38.5 mg, 0.07 mmol) in 1 mL of acetonitrile.The solution was subsequently

heated to 60ºC for 6 hours to induce cross-linking of the micellar core. The stability of the core-crosslinked polymer has been evaluated by comparing non-crosslinked and crosslinked polymers measured by DLS.

3.2.5 NIR responsive investigation

PEO-b-PFMA functional copolymer (50 mg, 0.013 mmol of furfuryl moieties) and the selenium-containing crosslinker (38.5 mg, 0.07 mmol), and ICG 10 in 1 mL of acetonitrile. The solution was subsequently heated to 60ºC for 6 hours to induce cross-linking of the micellar core. Then the solution was diluted to 4 mL, and irradiated with near-infrared source beam (708 nm 2mW). The stability of the core-crosslinked polymer has been evaluated by comparing non-crosslinked, crosslinked with ICG after and before irradiation measured by DLS.

3.2.6 Characterization

^{1}H NMR spectra were recorded on a JNM-ECP 600 (JEOL) instrument. Gel permeation chromatography (GPC) was performed using an HP 1100 apparatus, with DMAc with 50 mmol commercial polystyrene standards. Fourier transform infrared (FT-IR) spectra were measured on an Agilent Cary 640 spectrometer in the 4000–400 cm-1 spectral region. The samples were finely ground, mixed with spectroscopic grade potassium bromide (KBr) and pressed into pellets. For transmission electron microscopy (TEM; JEOL JEM-2010), the samples were prepared by depositing a drop of dispersed nanoparticles in water (upper portion) on copper grids. No staining was applied to the sample. Dynamic laser light scattering (DLS) measurements for determining the average hydrodynamic diameter of polymeric micelles were performed using an electrophoretic light scattering lithium chloride as a solvent at 50oC and an elution rate of 1 mL/min. The columns were calibrated with an instrument (ELS-800 Otsuka Electronics Corporation), equipped with an ELS controller, and a He–Ne laser at a

wavelength of 632.8 nm. The intensity of scattered light was detected at 90^{o} to an incident beam.

3.3 Result and Discussion

3.3.1 Preparation of PEO-Br macroinitiator

Water-soluble PEO-Br was synthesized in dichloromethane by reacting PEO5k and 2-bromoisobutyryl bromide in the presence of trimethylamine and DMAP. The yields in this typical esterification exceeded 90% with high purity. The ^{1}H NMR spectra of the macroinitiator were shown in figure 3.1B The analysis of the spectra was based on a comparison of the integrals of the ether methyl group (3.61 ppm). The methyl group from the 2-bromoisobutyryl moiety appeared at 1.90 ppm, and a new peak from the ester carbonyl bond (4.29 ppm) indicated that the esterification reaction was efficient. The peak integration ratio of c, a, and d was 2;3;6 (Figure 3.1A). The molecular weight of the macroinitiator was estimated to be Mw=5300 g/mol by the ^{1}HNMR result. This result had a correlation with the GPC result, Mw = 5304 g/mol, Mn = 5306 g/mol with Mw/Mn = 1.05 (Figure 3.2). FT-IR spectroscopy was used to identify representative functional groups in the macroinitiator (Figure 3.3B). After esterification, the carbonyl stretching band appeared at 1735 cm-1. The bands at 963 and 843 cm-1 were assigned to the characteristic of the crystalline phase of PEO [13]. The above data illustrated that the PEO-Br macroinitiator was successfully prepared with high purity

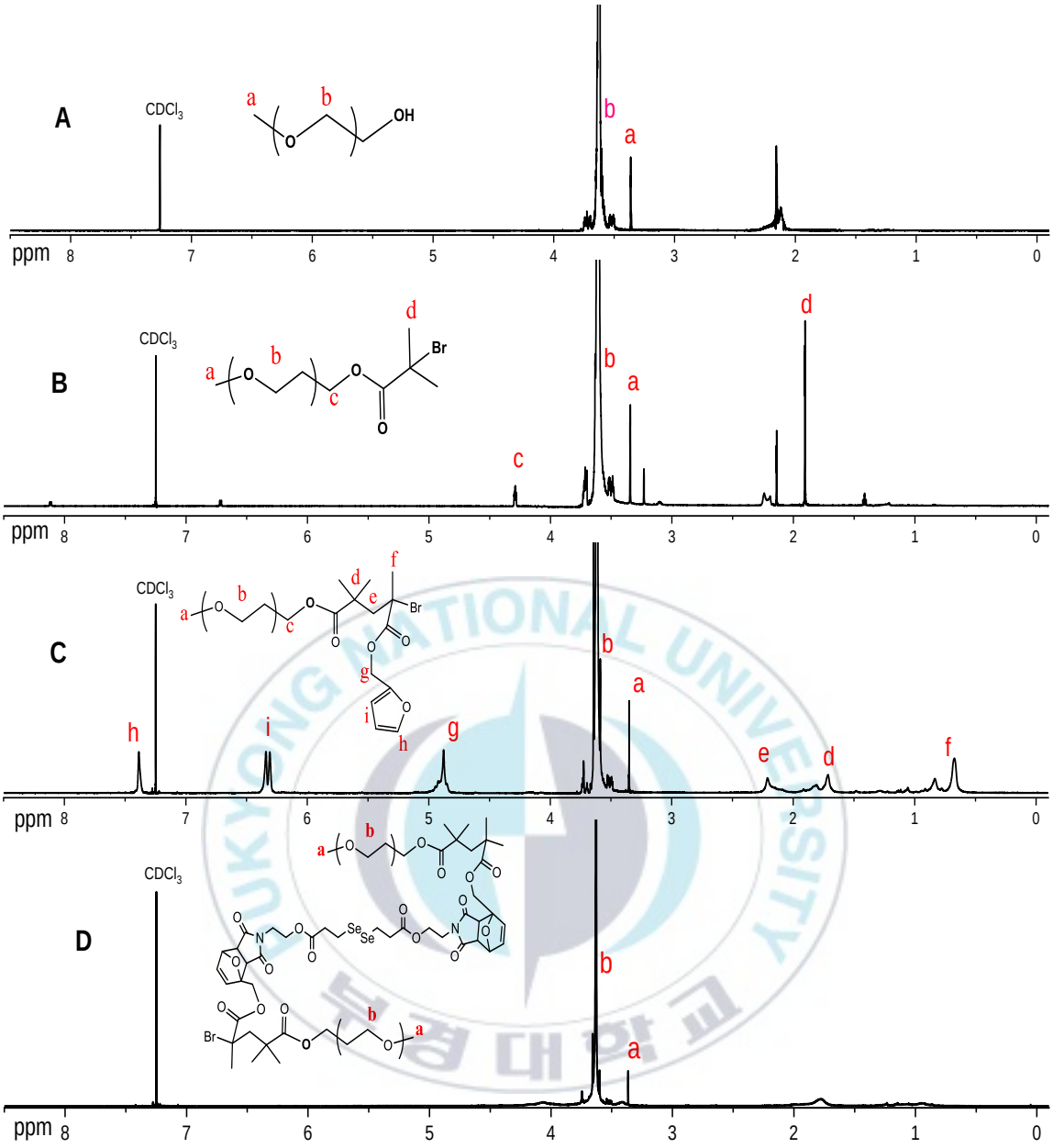


Figure 3.1 1H-NMR spectra of (A) PEO, (B) PEO-Br macroinitiator, (C) PEO-b-PFMA, and (D) core-crosslinked PEO-b-PFMA

3.3.2 Synthesis PEO-b-PFMA via SET-LRP

In this work, the amphiphilic block copolymer of PEO-b-PFMA was prepared by single electron transfer living radical polymerization (SET-LRP). This kind of radical

polymerization was reported very effective at room temperature and effective to control PDI[14]. Hydrophilic PEO-Br macroinitiators were polymerized with hydrophobic PFMA monomers in the presence of Me_6TREN as a ligand and copper wire as a catalyst under a nitrogen atmosphere. Similar molecular weights of constituting blocks were selected for this block copolymer. The PEO-*b*-PFMA was synthesized according to the literature as described elsewhere[15,16] with a good yield and well-defined parameters.

^{1}H NMR was used to confirm the formation of the block copolymer (Figure 3.1c). The proton of the methylene group in the main chain of PEO was shown at 3.63 ppm (*b*). The furfuryl moiety was introduced well in the polymer chain as the peak at 4.89 ppm (*g*) was assigned to the furfuryl methylene proton. The peak of the furan ring was shown at 7.40 ppm (*h*) and 6.35 ppm (*i*). The molecular weight of the block copolymer was confirmed by GPC and ^{1}HNMR. The polydispersity of PEO-*b*-PFMA (M_w/M_n) was 1.26 with M_w of 10300 g/mol and M_n *of* 8174 g/mol. This result was comparable with the estimated M_n value of 10285 g/mol from the ^{1}H NMR analysis. As confirmed by FT-IR spectra, ester and ether bands of PFMA moiety appeared at 1735 and 1115 cm^{-1}. Compared with the macroinitiator, the ester bond in the copolymer at 1735 cm^{-1} was much stronger. Furan rings in PEO-*b*-PFMA produced vibrational bands at 753 and 607 cm^{-1} (Figure 3.3c).

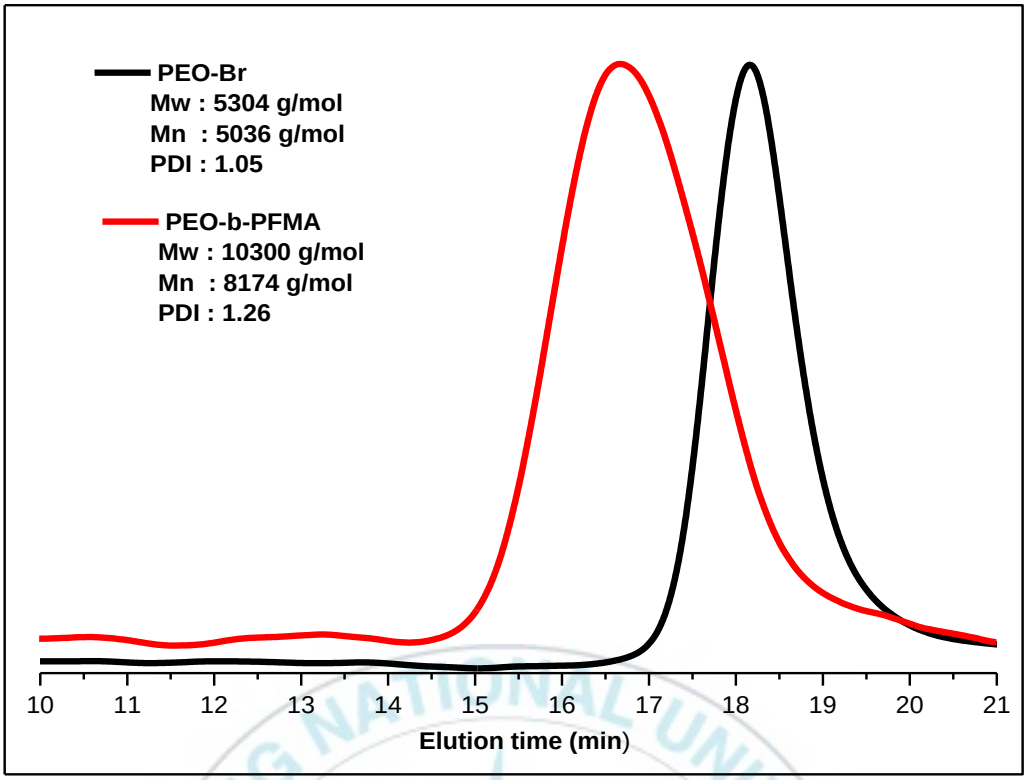


Figure 3.2 GPC chromatogram of PEO-Br and PEO-*b*-PFMA

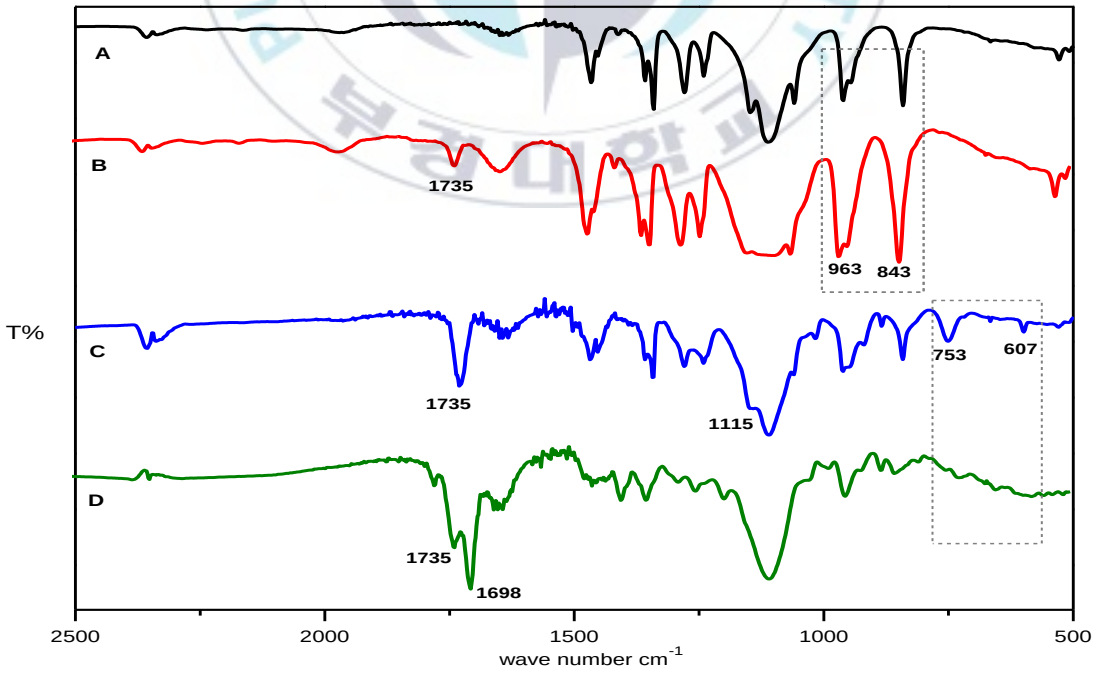


Figure 3.3 FT-IR spectra of (A) PEO, (B) PEO-Br macroinitiator, (C) PEO-b-PFMA, and (D) core-crosslinked PEO-b-PFMA

3.3.3 CCL micelles of PEO-PFMA block copolymer by Diels- Adler click chemistry

Diels-Alder click reaction was utilized to prepare core-crosslinked micelles from PEO-*b*-PFMA. In this work, the hydrophobic segment from amphiphilic PEO-PFMA was crosslinked with the diselenium containing bismaleimide crosslinker. The ratio between the furfuryl moiety and the crosslinker was 2:1 molar equivalent. Encapsulation of the crosslinker into the core of the amphiphilic block copolymer was done in acetonitrile then the solution was heated up to 60^{o}C for 6 hours to induce Diels Alder reaction. Subsequently to obtain micellar solution water was added dropwise to the core-crosslinked solution.

Dialysis against water was used to remove the excess amount of acetonitrile in the micellar solution. The micelle size of the core-crosslinked polymers and non-crosslinked were evaluated by DLS. Non-crosslinked polymeric micelles were prepared by the same procedure without heating. Non-crosslinked micelles and core-crosslinked micelles in water were compared and show the different diameter around 88 nm (Figure 3.4 a-b). After Diels-Alder reaction the size of micelles increase. This phenomenon might be due to the inclusion of bismaleimide crosslinkers into the core of micelles and rearrangement caused by [4+2] Diels-Alder cycloaddition reaction.

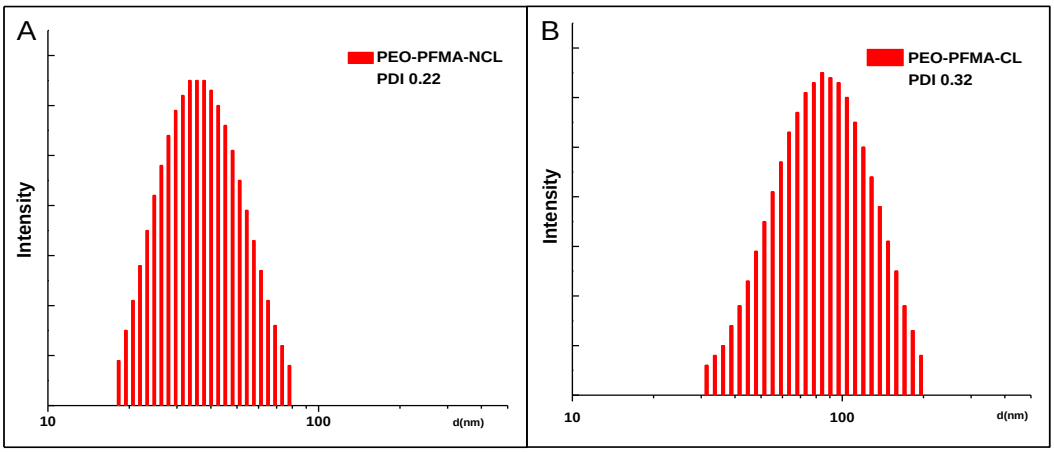


Figure 3.4 DLS spectra of (A) non-core-crosslinked micelles, (B) core-crosslinked micelles

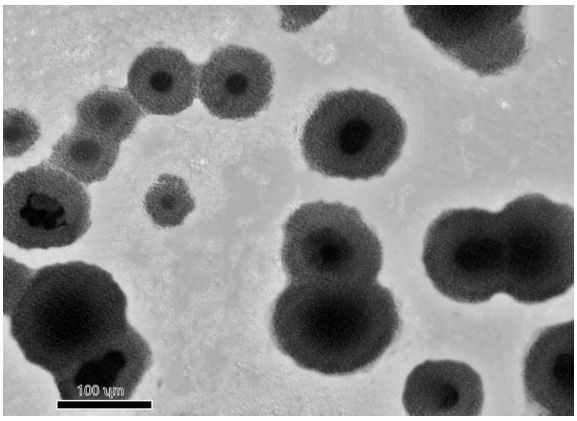


Figure 3.5 TEM Image of core-crosslinked micelles

The morphology of micelles was confirmed by TEM, spherical shape with the size in agreement with the DLS result (Figure 3.5). Another evidence of the crosslinking reaction was revealed by ^{1}H-NMR and FT-IR. As shown in the ^{1}H-NMR spectra of core-crosslinked polymer (Figure 3.1d), the resonance of furfuryl protons disappeared whilst the protons of the methylene in the PEO group still remained, which indicated that PFMA blocks were cross-linked in the core and PEO blocks formed the outer shell. The new band in the FT-IR spectra appeared at 1698 cm-1, which was assigned to the vibration of maleimide in the crosslinked copolymer. Decreasing in the furan absorption band at 753 and 607 cm-1 after Diels-Alder reaction proved also the crosslinking reaction

3.3.4 NIR responsive investigation

Diselenide bond can be cleaved not only by hydrogen peroxide but also by ROS such as singlet oxygen[6].ICG that exhibits maximum absorption at around 780 nm has been proved the capability of generating ROS under NIR light irradiation, thus ICG was loaded into core crosslinked polymeric of PEO-PFMA as NIR-responsive micelles. ICG is a zwitterionic

amphiphilic molecule, which strongly adsorbs to both hydrophilic and hydrophobic surfaces, it can be easily encapsulated into diselenide- cross-linked micelles. The degradation of micelles solution was studied by DLS measurement (Figure 3.6). ICG-loaded micelles were diluted in acetonitrile with a concentration of micelles 12.5 mg/mL. NIR light (780 nm, 1mW) was applied for 1-2 hours. An obvious decline intensity down could be observed after the first irradiation, and then it dropped to lower intensity of micelles. respectively, after the following two irradiations. The periodic irradiations of NIR light yielded a turbidity decrease step by step that was indicative of a stepwise disassembly of micelles. The insert photographs in Figure 3.8 show that the micelles before irradiation present a deep green solution, in which course they turned into a transparent solution after irradiations, suggesting the degradation of ICG.

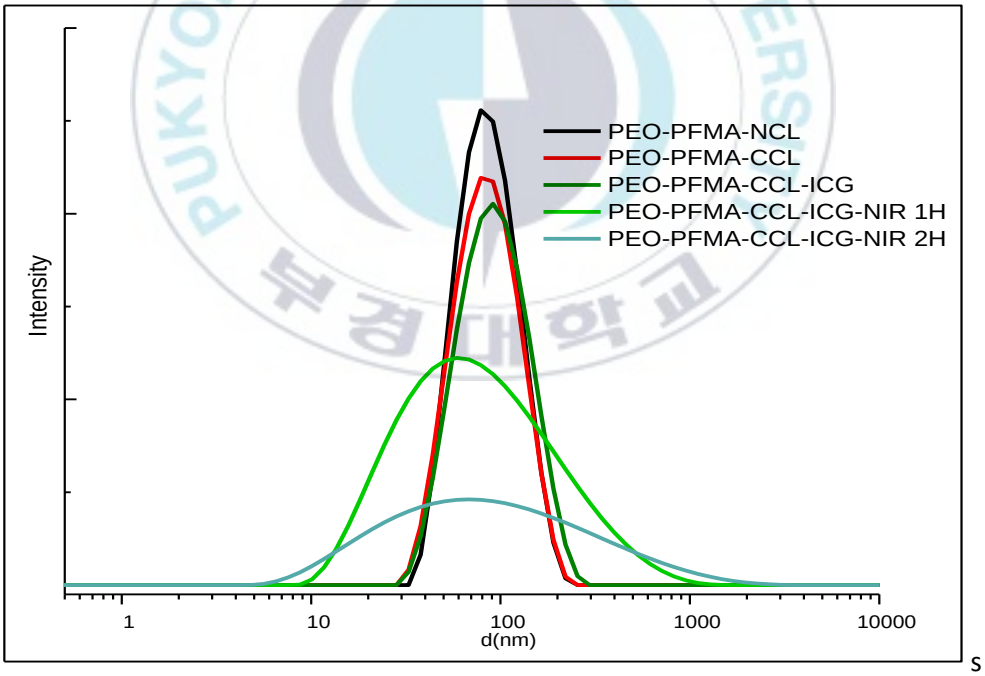


Figure 3.6 The degradation of micelles induced by NIR

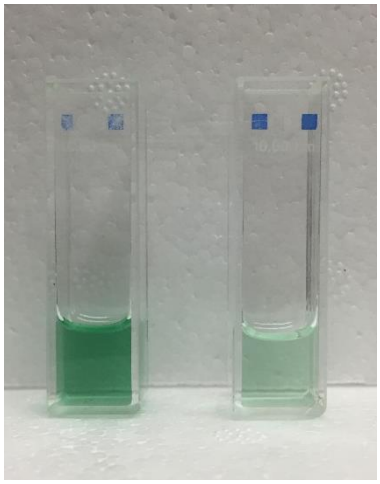


Figure 3.7 The degradation of micelles solution after and before irradiation

3.4 Conclusion

The new responsive diselenide containing crosslinker was successfully synthesized. The Diels-Alder reaction was selected for designing reversible crosslinking structures triggered by redox conditions and NIR irradiation. The core-crosslinked micelles based on the amphiphilic block copolymer of PEO-PFMA was synthesized by the SET-LRP method. The crosslinking reaction had been achieved by co-solvent micellization strategy, resulting in the average size of micelles below 100 nm. A novel type of reversible hydrophobic core-crosslinked micelles was developed and it would be promising for application in drug delivery system.

3.5 Reference

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