MULTIFUNCTIONAL NANOGELS VIA SELF-ASSEMBLY OF POLYMERS

by

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To my family

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ABSTRACT

Water soluble multifunctional nano structures are promising candidates as imaging agents for early and post-treatment diagnostics. In this thesis, design and synthesis of novel multifunctional nanogels have been achieved by chemically crosslinking nanogels obtained via polymer self-assembly. A poly(ethylene glycol)methacrylate (PEGMA) based maleimide bearing polymer was synthesized via atom transfer radical polymerization (ATRP). In particular, a PEGMA containing amphiphilic copolymer was used to synthesize the nanogels due to its lower critical solubility temperature (LCST) behavior. These polymers form nano-size aggregates in aqueous solutions upon heating. Due to the presence of thiol-reactive maleimide units on these polymers they can be crosslinked via thiol bearing crosslinkers to obtain nanogels. After gelation, functionalization of residual maleimide groups in the nanogels are available for conjugation of imaging agents, as shown via addition of a thiol bearing fluorescent dye (BODIPY-SH). In addition, to demonstrate multifunctionality of nanogels, thiol bearing cyclic peptide based targeting group, cRGDfC, and maleimide bearing dye N-(fluoresceinyl) maleimide were sequentially conjugated to nanogels under mild conditions. Parent nanogels did not show any *in vitro* toxicity on cancerous breast cell lines. Presence of the targeting group on the nanogels increased the internalization of nanogels into the cancerous breast cell lines as deduced from fluorescence microscopy and flow cytometry studies. Overall, facile synthesis of a multifunctional nanogel that can serve as a promising candidate in the field of diagnostics was achieved.

ÖZET

Suda çözünen, çok işlevli nano yapılar görüntüleme ajanı olarak tedavi öncesi ve sonrasında diagnostik alanlarda kullanım açısından gelecek vaat etmektedir. Bu tezde, çoklu işlevi olan nanojeller kendiliğinden düzenlenen polimerlerin kimyasal şekilde çapraz bağlanmasıyla sentezlenmiştir. Poli (etilen glikol) metakrilat bazlı, maleimid taşıyan polimerler atom transfer radikal polimerleşmesi (ATRP) yoluyla sentezlenmiştir. Bu amfifilik polimerler alt kritik çözünme sıcaklığı özellikleri nedeniyle nanojel sentezinde kullanılmışlardır. Bu polimerler sulu çözeltide ısıtıldığında agregalar oluşturduğu için, tiyol bulunduran çapraz bağlayıcılar yoluyla çapraz bağlanarak iyi tanımlanmış boyutlarda nanojeller elde edilebilir. Jelleşme sonrasında, artan maleimid gruplarının işlevselleştirilmesi tiyol taşıyan hidrofobik boyanın (BODIPY-SH) eklenmesi ile gösterilmiştir. Ek olarak, nanojellerin çoklu işlevselliğini göstermek için, tiyol bulunduran siklik peptit bazlı hedefleme grubu, cRGDfC, maleimide taşıyan boya N-(Floresinil) Maleimid sırasıyla nanojellere hafif koşullarda conjuge edilmiştir. Nanojeller meme kanseri hücre hatları üzerinde citotoksisiteye neden olmamıştır. Nanojeller üzerinde hedefleme grubu bulunması, nanojellerin meme kanseri hücre hatlarına girmesini kolaylaştırmıştır. Sonuç olarak, kolay sentezlenen, çoklu işlevli ve iyi tanımlanmış boyutlarda nanojeller tasarlanmıştır ve bu nanojeller diagnostik alanda gelecek vaat etmektedir.

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LIST OF ACRONYMS/ABBREVIATIONS

ATRP	Atom Transfer Radical Polymerization
$CDCl_3$	Deuterated Chloroform
CH_2Cl_2	Methylene Chloride
DLS	Dynamic Light Scattering
EPR	Enhanced Permeability and Retention
FT-IR	Fourier Transform Infrared
GPC	Gel Permeation Chromatography
LCST	Lower critical Solubility Temperature
MeOH	Methanol
NG	Nanogel
NMR	Nuclear Magnetic Resonance
P	Polymer
PDI	Poly Dispersity Index
PEGMEMA	Poly(ethylenglycol)methacrylate
RT	Room Temperature
TEA	Triethylamine
TEM	Transmission Electron Microscopy
THF	Tetra Hydro Furan
UV	Ultraviolet

1. INTRODUCTION

1.1. Nanomaterials for Bioapplications

Particles having size smaller than 100nm are generally accepted as nanomaterials which are promising candidates for applications in controlled drug delivery, biosensing, tissue engineering and bioimaging [1]. The high performance of nanomaterials for the above mentioned areas are caused by their distinct physical characteristics such as their 3D shapes and high surface over mass ratio, therefore they contain highly functionalizable surface which allows them to react, adsorb and transport more efficiently. In order to formulate nanomaterials, a wide variety of chemically diverse materials can be used. Natural products like dextran, hyaluronic acid, chitosan, lipids and phospholipids, or synthetic polymers, silicon and metals have been utilized to formulate nanomaterials depending on the application [2].

Liposomes, nanoparticles, nanogels, nanotubes, quantum dots and micelles are the most frequently used types of nanomaterials which allow facile surface modification (Figure 1.1) [3]. Modifications on the surfaces of nanoparticles which have the size similar to biomolecules serve as excellent probes for various bioapplications. Surface modifications facilitate their solubility in aqueous medium, increase their biocompability and allow biosensing.

The nanomaterials can be tuned to achieve targeting, respond to physiological stimuli (pH, temperature or redox) and embody imaging characteristics which makes them suitable candidates for various biomedical and pharmaceutical applications [4]. In order to display these features, polymers are widely used as either the core material or as an outer shell of inorganic nanomaterials. Among various nanomaterials, the formation of nanogels and polymeric nanoparticles involve convenient and straightforward synthesis methods to obtain various nanomaterials that either interact or respond or do both in biological settings [5].

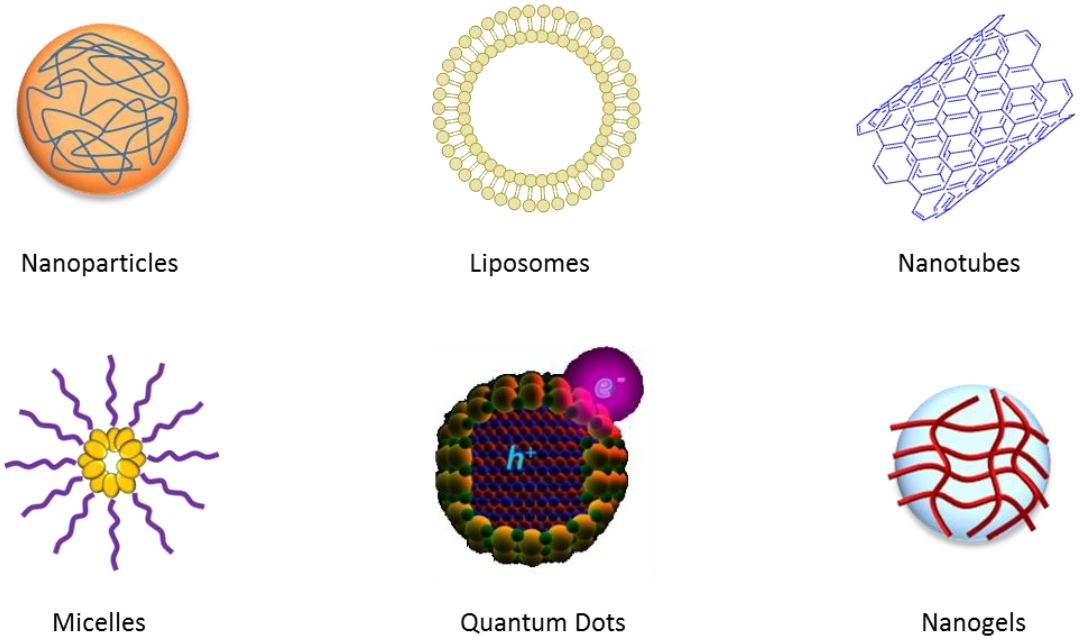

Figure 1.1. Various nanoparticles for bioapplications

1.2. Polymeric Nanoparticles for Bioapplications

Throughout the last few decades, usage and development of polymeric nanoparticles has extensively increased in biological applications, especially for controlled and targeted delivery purposes. Interest on the utilization of polymeric nanoparticles for drug delivery have witnessed this increase because it can increase the therapeutic effect and overcome the limitations of liposomes, a class of nonmaterial that is currently used in clinical formulations. Although, liposomes can be used for targeted delivery with lower drug degradation and toxic effects, they often suffer from rapid leakage and lower encapsulation efficiency compared to polymeric nanoparticles [6]. Moreover tailoring of polymeric nanoparticles, both their surface and shell part, is easier due to versatility, modularity and variety of polymers.

Most of the widely reported polymeric nanoparticles consist of natural products like hyaluronic acid, dextran, or synthetic polymers like poly (D,L -lactic acid) , (PLA), poly(D,L-lactide-coglycolide) (PLGA), and poly(caprolactone), (PCL) because of their biodegradability [7,8]. Nanoparticles have specific passive targeting ability for tumor tissue through the large pores caused by the disordered vasculature and lymphatic drainage. This characteristic is referred as the enhanced permeability and retention (EPR) effect. Biodegradable nanoparticles can carry their cargo to tumor tissue and release their cargo

after degradation in the cells. Moreover, the solubility and blood circulation of the nanoparticles are enhanced by conjugating PEG chains to their surface [9].

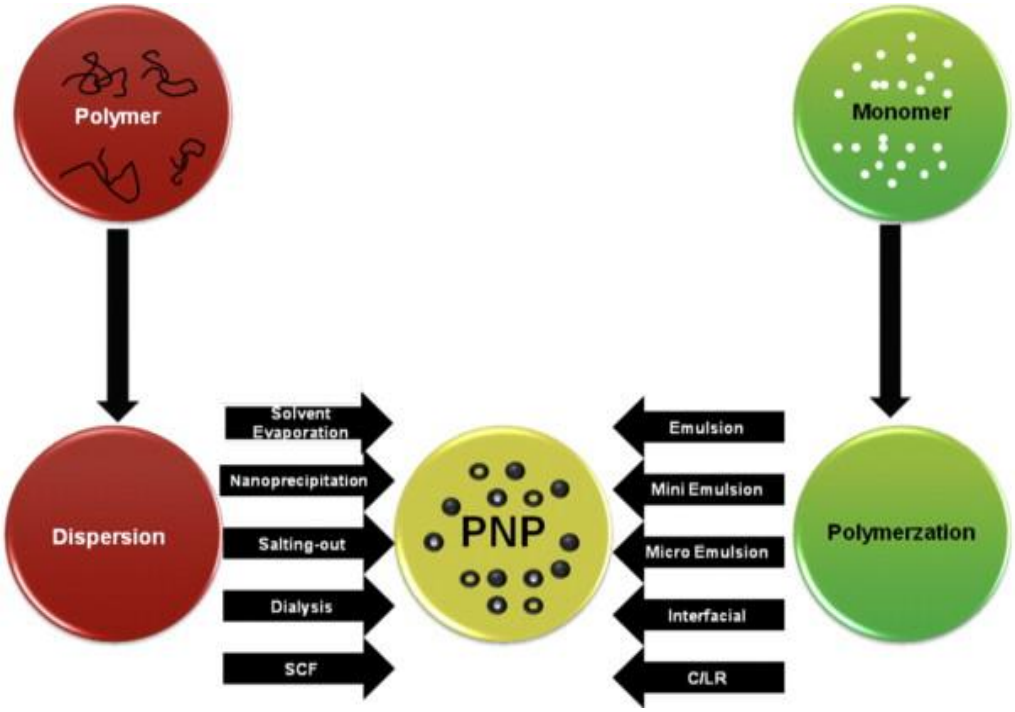

Figure 1.2. Polymeric nanoparticle formation methods. Reprinted with permission from [10].

Most common methods to obtain polymeric nanoparticles through dispersed polymers are solvent evaporation, salting-out, nanoprecipitation and dialysis (Figure 1.2.). Solvent evaporation method uses a volatile solvent system, where after evaporation of one of the solvent the polymers are trapped in the other solvent as mini-emulsions [11]. Salting-out uses a solvent system consist of water and water-miscible solvent and high concentration of salt emulsification is achieved [12]. Nanoprecipitation generally employs water and a water miscible low boiling solvent. Diffusion of polymers in water is rapid, thus it favors formation of polymeric droplets [13]. Dialysis is another widely used method in which the polymer is dissolved in an organic solvent and dialysis is performed against a solvent which is not favored by polymer. After displacement of the solvent homogeneous nanoparticles can be obtained [14].

1.3. Nanogels for Bioapplications

Nanogels are nanosized three dimensional polymeric meshes which are formed by physical or chemical crosslinking of monomers or polymers. Nanogels are generally used for tissue engineering, biosensing and mainly for drug delivery purposes like most of the other nanomaterials. Nonetheless, ordinary polymeric nanoparticles contain hydrophobic parts of polymers densely packed in the core and free volume increases towards the shell. On the other hand, nanogels can have a more homogeneous interior network compared to polymeric nanoparticles [15]. Formulation using hydrophilic building blocks affords nanogels that are amenable to diffusion of small molecules into and out of these structures.

Sizes of nanogels are tunable depending on their formation method. Drugs, cell targeting ligands and contrast agents can be covalently attached to the surface of the nanogels. Nanogels can be engineered to be biodegradablewhen synthesized using biodegradable polymers or biodegradable crosslinkers. Upon arrival to the target they can release their cargo quickly and cause a drastic increase in the local concentration of drugs. Disulfides, peptides, anhydrides, esters and acetals are widely used linkages to tailor biodegradability into nanogels [16].

Drug delivery agents always aim to have high encapsulation efficiency, however high encapsulation efficiency does not guarantee that a nanomaterial can be used safely and effectively. If the cargo is not stably encapsulated, it can cause leakage which will damage the healthy tissue and have minor impact on tumor tissue. Thus, the stability of the nanogels conjugates make them more advantageous than the self-assembled non-crosslinked nanoparticles, since the stability enables nanogels to carry their cargo to the site of action at a concentration within the therapeutic window [17].

Encapsulation stability is important for nanogels. However, upon desire a nanogel system should be able to release their cargo at the target in addition to stability during circulation in the body. Thus nanogels should be stimulus responsive. Those responses can be obtained by forming nanogels with chemical bonds which may be pH, temperature, redox, light reactive or cleaved by enzymes [18].

Size control is another important issue to have desirable nanogels for drug delivery purposes. In order to passively target tumor tissue and utilize EPR effect, sizes of the nanogels should be more than 20 nm. Nanogels among that size have the passive targeting ability due to disarranged cellular structure in the tumor tissue. Moreover, sizes more than 200 nm is not desirable for nanogels since sizes higher than 200 nm can limit the efficient endocytosis of particles into the cells [19].

In addition to passive targeting caused by the EPR effect, nanogels can undertake active targeting by incorporating antibodies or receptor binding ligands to their surface. Surface recognition facilitates selective delivery of the cargo to the target. For instance, Lyon and coworkers used folic acid as a ligand which has high affinity for folic receptors overexpressed in some types of tumors. They designed a poly (N-isopropylacrylamide) (pNIPAm) based nanogel with the N,N0 -methylenebis(acrylamide), biscrosslinker and folic acids were conjugated to amine groups on the nanogels surface which increased the efficiency of nanogels in siRNA delivery [20].

Toxicity and anti-biofouling character is a must for a nanogel to be used in the drug delivery. Most of the nanogel systems have an acrylic or methacrylic core which are known to be harmless and generally hydrolyzed into small alcohols and methacrylic acid. Moreover, nanogel systems generally use non-toxic oligo ethyleneoxide based monomers to favor self–assembly behavior and antibiofouling effect [21]. Another non–toxic and biodegradable group is disulfides which can be reduced wtih the help of glutathione molecules in the body. Thus disulfide bearing crosslinkers are widely used for the biodegradable nanogel synthesis. For instance, Li and coworkers designed a nanogel system with oligo ethylenoxide and six-member cyclic ortho ester group bearing acrylates and disulfide containing bisacrylate with the help of miniemulsion polymerization. When they add dithiothreiotol (DTT) or decrease the pH, nanogels release their cargo after degradation [22].

1.4. Synthesis of Nanogels using Surfactants

In order to synthesise nanogels, there are two main approaches. The first one involves polymerization of monomers with a crosslinkable monomer which has at least two polymerizable functional units. Monomers with bisfunctional groups will crosslink the

polymers during polymerizations. In order to, prepare gels from monomers usual approach involve using miniemulsions or inverse miniemulsions to trap monomers in a nanosized emulsion. Thus, polymerization undergoes in the nanosized emulsions and thus controls the size of the nanogels [23]. For example, Matyjaszewski and coworkers formed inverse mini-emulsions by using the surfactant Span80 in water and cyclohexane. Within these miniemulsions they polymerize polyethylenglycol methacrylates with a disulfide bearing polyethylengycol dimethacrylate crosslinker. They perform Inverse Miniemulsion ATRP to obtain biodegradable nanogels (Figure 1.3.) [24].

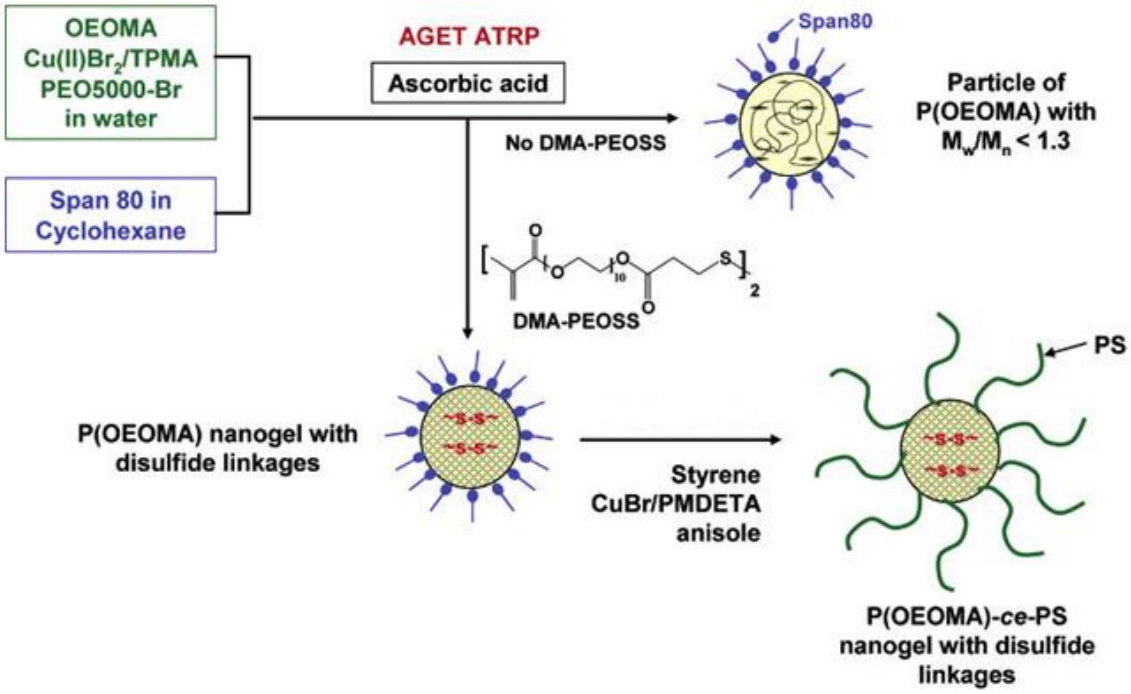

Figure 1.3. Nanogel synthesis by PEGMA monomers by using AGET ATRP in the inverse mini emulsions. Reprinted with permission from [24].

Second approach to synthesize a nanogel involves crosslinking of polymers via a crosslinker. In order to synthesize the nanogel in a controlled manner, stable aggregates are necessary just like the synthesis of nanogel by crosslinking monomers. Polymers in miniemulsions will have a high local concentration, therefore facilitate the gelation despite their low total concentration. Mini-emulsion technique can again be used for the synthesis of nanogels [25]. Surfactants can trap polymers as mini emulsions or inverse miniemulsions in aqueous or oil phase and yield nanogels (Figure 1.4.). For instance, Moeller and coworkers

formulated a nanogel system by crosslinking polymers in inverse miniemulsions. They use a star shaped poly (ethylene oxide-stat-propylene oxide) (sP(EO-stat-PO)) and linear poly(glycidol) (PG) bearing grafted thiols. They formed inverse minielmusions in the presence of span 80 and tween 80 in a mixture of PBS buffer and hexane. After oxidation of thiol groups they obtain biodegradable nanogels crosslinked through disulfides within the inverse miniemulsions [26].

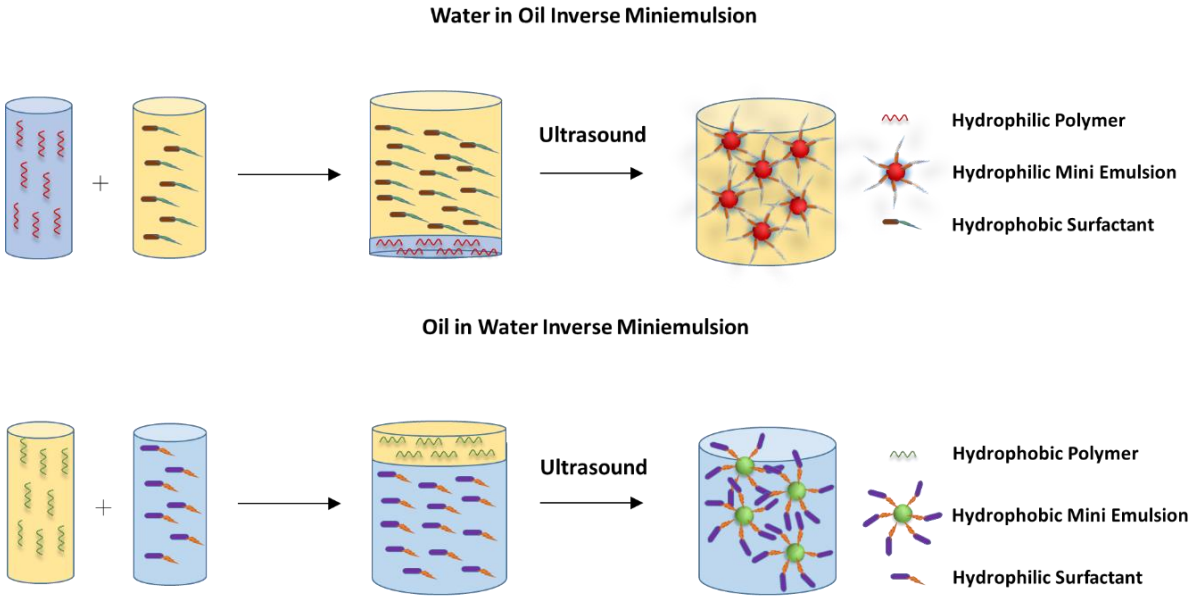

Figure 1.4. Nanogel synthesis via polymers in miniemulsion and inverse miniemulsion

1.5. Synthesis of Nanogels via Self – Assembly

Use of surfactants enables synthesis of nanogels via polymers in a controlled manner with a narrow size. Nonetheless, removal of those surfactants after the synthesis is a challenge and even the trace amounts of those surfactant can affect the applications of nanogels *in vitro* and *in vivo* systems. On the other hand, self – assembly characteristics of associating polymers can be used to obtain stable polymeric aggregates for the synthesis of nanogels [27]. Polymers containing groups with charges or hydrogen bonding ability can associate in the water. Although, those physical bonds are weak forces, when many of those groups are gathered in a small volume they possess quite a strong interaction [28]. Due to the lower critical solubility temperature (LCST) behavior of the temperature responsive hydrophilic polymers, above a certain temperature solubility of those polymers decrease greatly. Therefore, they start to form stable aggregates in the aqueous solutions due to the

strong interactions among those polymers. [29]. Using self-assembling polymers for the synthesis of nanogels is advantageous because they can form stable aggregates above their LCST and remove the necessity of surfactants to form stable aggregates (Figure 1.5.).

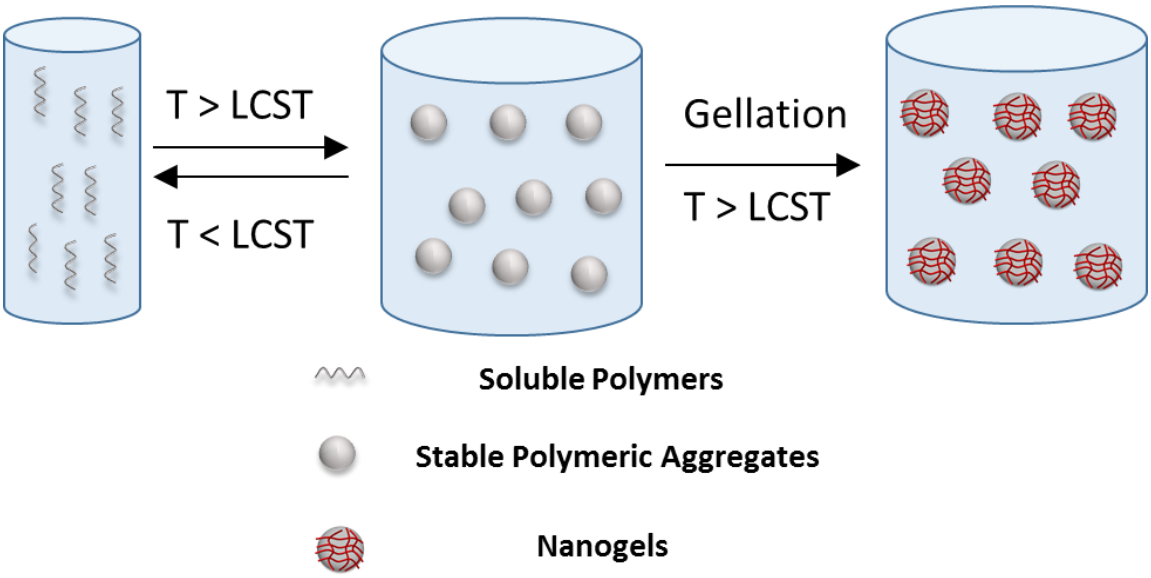

Figure 1.5. Nanogel synthesis via polymers after their self–assembly above their LCST

N-isopropylacrylamide (pNIPAm) is one of the most extensively used polymers in the biomedical applications since it has a LCST temperature at 32^{o}C which enables structural switches between room temperature and body temperature [30]. In recent years, use of derivatives of poly (2-oxazoline), polyethers and poly vinyl alcohols in biomaterials was increased to utilize their self–assembly behavior. Addition of these polymers add thermosensitive characteristics to the materials and enable self–assembly above their LCST. [31].

Disulfide formation is one of the most extensively used methods for the crosslinking of polymers to obtain nanogels via self-assembly. For instance Thayumanavan and coworkers used a polymeric system including polyethyleneglycol (PEG) and pyridyl disulfide (PDS) units to form nano sized aggregates in the water. PEG groups add hydrophilicity and thermoresponsive characteristic to the polymer whereas pyridyl disulfides are the hydrophobic parts and can be converted to thiols with little amount of dithiotheitol (DTT). After heating the polymers above their LCST temperature, DTT is used

to form free thiols which lead to formation of disulfides among different polymers via oxidation and yields a disulfide bridged nanogels (Figure 1.6.) [32].

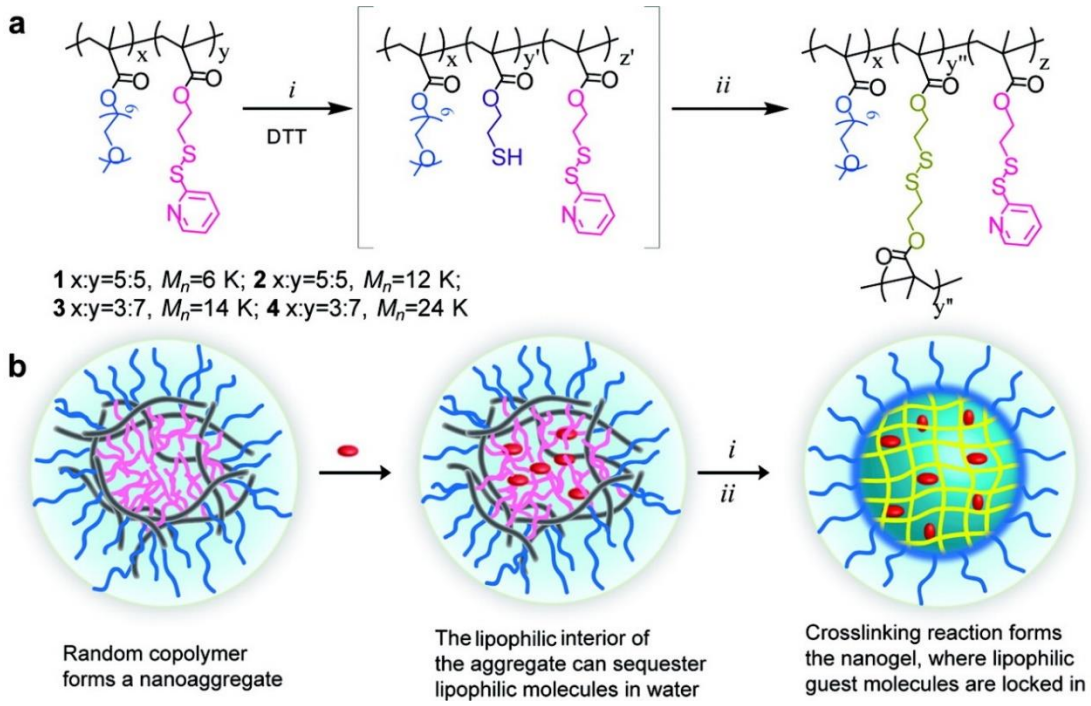

Figure 1.6. Nanogel synthesis after cleaving PDS groups of polymers with DTT and formation of interchain crosslinking among polymers. Reprinted with permission from [32].

Amine groups can be used for the synthesis of the nanogels because they can react with isocyanides, carboxylic acids and activated esters. Addition of diamine bearing crosslinkers can yield nanogels after obtaining self-assemblies of random copolymers or micellar assemblies of block copolymers [33], [34]. For instance, Davis and coworkers designed a block copolymer in which one block consists of PEG acrylate and other block consists of a random polymer of vinylbenzyl chloride and pentafluorophenyl acrylate. After obtaining a micellar assembly by changing the solvent from DMF to water, a diamine crosslinker is used to obtain nanogels [35].

Besides covalent bonding, physical bonds such as electrostatic, host–guest and hydrophobic interactions are used to form nanogels [36], [37]. Although, physically crosslinked nanogels do not possess the stability of chemically crosslinked nanogels, , they still can maintain their nanogel forms as micelles above a concentration [38]. Cholesterol bearing hydrophilic polymers such as mannan, chitosan, poly(amino acids) and pNIPAm are

used to form nanogels since the interaction between the hydrophobic cores are strong and enables formation of stable nanogels. Advantageous parts of the formation of physically crosslinked nanogels are not using the toxic surfactants, crosslinkers or catalyst during their preparation.

Bastos-González and coworkers prepared a physically crosslinked nanogel system with electrostatic interactions. Slow addition of tripolyphosphate (TPP) to a solution of chitosan yields nanogels. Although, it is a facile method to obtain hydrophobic nanogels, nanogels are not as stable as covalently crosslinked nanogels. They tend to fuse and form larger particles. Moreover, chitosan groups of the particles are degraded without the enzymes in an aqueous medium. On the other hand, this system is highly sensitive to pH changes and it can be disrupted in highly acidic or basic media [39].

1.6. Utilization of 'Click' Chemistry and Michael Addition in Nanogel Formation

In 2001, the concept of 'click' chemistry is defined by Sharpless to indicate modular, selective and efficient reactions for large and small scale applications. Reactions which are defined in the click chemistry group occur at mild conditions with high yields and produce byproducts which can be removed easily without chromatography. Moreover, the reactions are not sensitive to water and oxygen and can be performed even in water. These characteristics of 'click' type reactions boost their use in the field of polymers, macromolecules, and biomolecules. Thus, the macromolecules and biomolecules can be functionalized without damaging them thanks to 'click' chemistry [40].

'Click' chemistry is important especially for the synthesis of clickable nanogels because there has to be functionalizable groups on the nanogels after crosslinking for many applications. Thus, mild and selective 'click' chemistry is generally preferred for the synthesis of nanogels. For example, Haag and coworkers prepared a nanogel by using a two different type of poly vinyl alcohol (PVA). First one is functionalized with a carboxylic acid group and disulfide containing alkyne end group. The other polymer PVA is functionalized with azide groups. Two different polymer are mixed in different phosphate buffer saline (PBS) solution with $CuSO_4$ and mixed with acetone. Thanks to copper catalyzed Huisgen

click reaction triazole groups are formed between azides and acetylenes. Thus, pH sensitive, biodegradable nanogels are formed as in figure 1.7 [41].

Michael addition is very efficient reaction and it utilizes the coupling of nucleophiles with electron deficient olefins. Michael addition is selective, produces no byproduct and it can be used with a vast number of polymerizable monomers and functional precursors. Especially for the bioapplications where the concentrations are too low and temperature cannot be elevated, Michael addition is widely used for the conjugation of proteins without damaging any other groups. Nucleophiles like amine and thiol groups on the surfaces of proteins are easy targets for maleimide, acrylate and methacrylate groups [42]. Especially, thiol and maleimide conjugation is more effective with base catalysis as in figure 1.8. Therefore, this facile and selective reaction is widely used for the synthesis of nanogels.

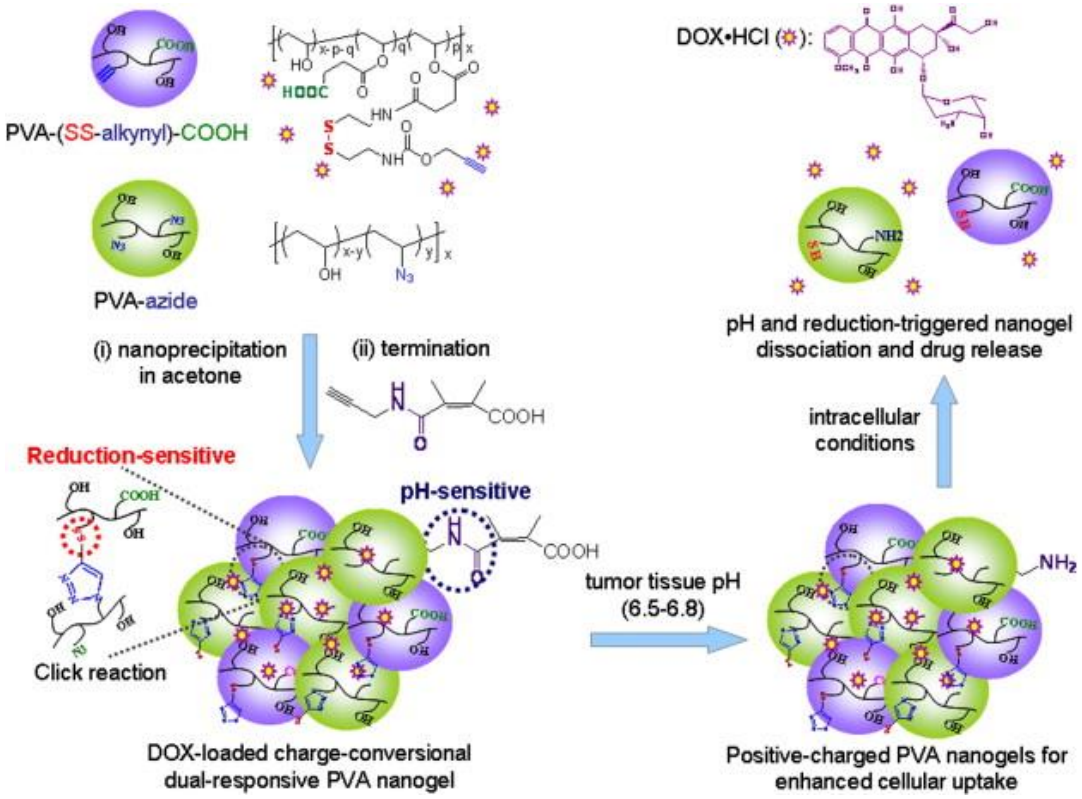

Figure 1.7. Nanogel formation via Huisgen click reaction. Reprinted with permission from [41].

For instance, Möller and coworkers prepared a six armed star shaped PEG which is terminated with acrylate groups. In order to crosslink this star shaped PEG, it is mixed with hen egg ovalbumin protein containing 15 lysine groups on its surface. By using a water in oil emulsion, stable aggregates of the polymers and proteins are obtained. Thanks to

accessibility of amine groups of lysines, acrylate groups of PEG polymers interact with them easily and perform a Michael addition to obtain nanogels [43].

Another approach to synthesize nanogels is made by Kazunari and coworkers by using a modified pullulan. They have used a pullulan which has cholesterol and acrylate groups. The pullulans are dissolved in PBS and mixed with a four armed PEG polymer terminated with thiols groups. Michael addition can be performed easily between acrylate and thiol groups. The mixture is put in polytetrafluoroethylene (PTFE) membranes and kept there for 1h to obtain disk–shaped nanogel crosslinked hydrogels. Those nanogel including hydrogels have 10 times more storage capacity thanks to amphiphilic nanogels in the core [44].

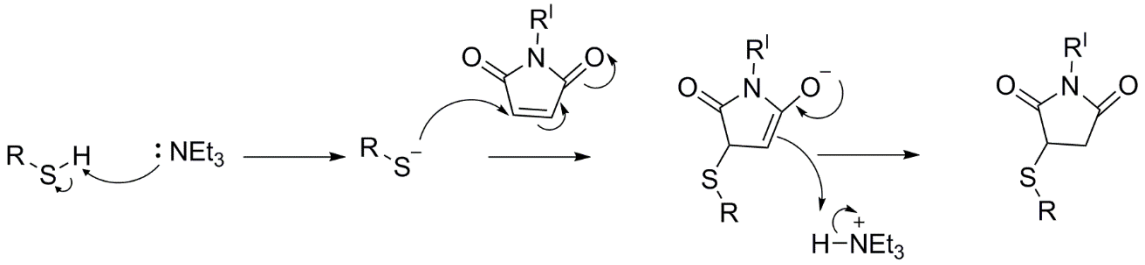

Figure 1.8. Thiol – Maleimide Michael addition reaction in presence of Et_3N catalyst.

1.7. Atom Transfer Radical Polymerization (ATRP)

ATRP is one known as one of the most powerful and robust polymerization techniques for the rapid synthesis of high molecular weight poly (methacrylates). It proceeds by first a homolytic cleavage of a halogen from an initiator by a metal complex in a lower oxidation state. It is widely performed by using a tetradentate Cu^I complex with a tertiary alkyl bromide. During initiation the process metal complex go to a higher oxidation step with the addition of halide and a radical is formed on the initiator [45]. After that this radical can attack to monomer and start the propagation of polymer. Halide bound the oxidized state of metal can be transferred to end of the propagating polymer which is the controlling cap of ATRP as in figure 1.9. Therefore, ATRP can be used to synthesize complex polymers with low poly dispersity and target molecular weight [46].

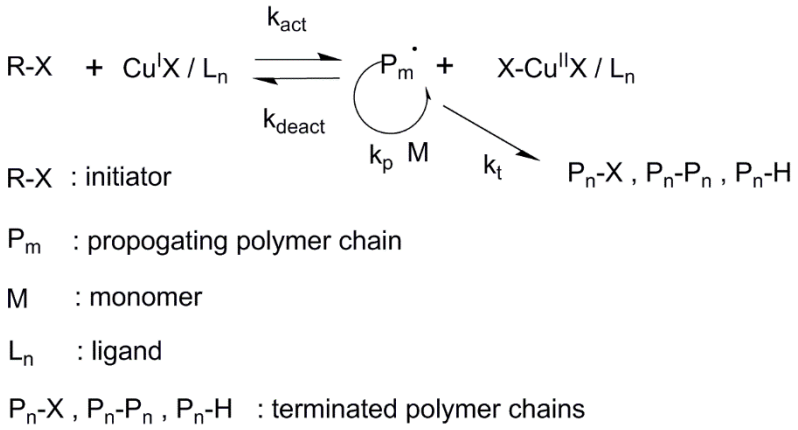

Figure 1.9. Mechanism of ATRP

2. AIM OF THE STUDY

The aim of this study is to synthesize multifunctional and well-defined nanogels for application in targeted cellular imaging. Nanogels can be synthesized using a maleimide bearing poly(ethylenglycol)methacrylate based amphiphilic copolymer due to its LCST behavior via nucleophilic thiol-ene reaction. Above the LCST of polymers, they form stable aggregates, which enables their crosslinking in very dilute concentrations. Unreacted maleimide and leftover free thiols on nanogels can be further functionalized with targeting groups and fluorescent dyes (Figure 2.1). Cellular targeting and internalization of these nontoxic fluorescent nano structures can be enhanced by the addition of a peptide based targeting group.

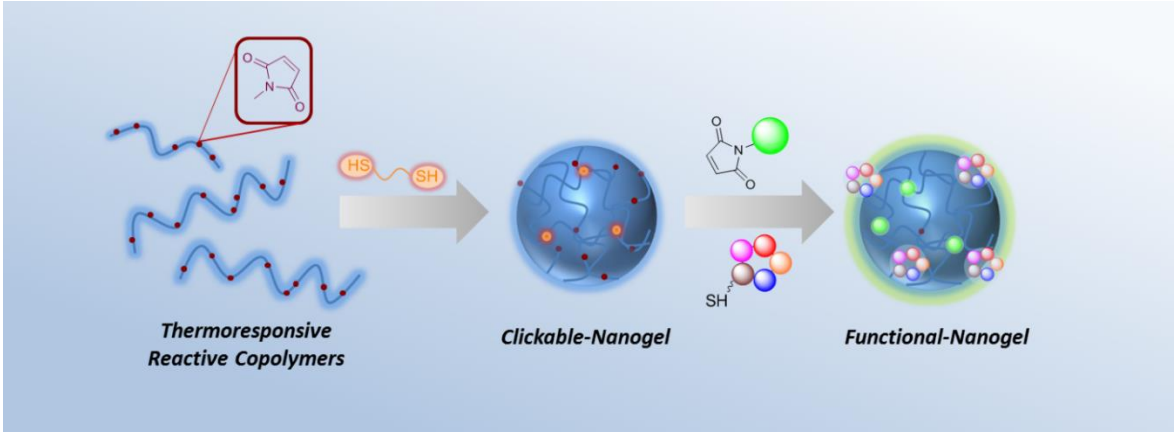

Figure 2.1. General scheme of the project

3. RESULTS AND DISCUSSION

3.1. Design and Synthesis of Thiol Reactive Polymers

For the bioconjugation of small peptide groups to the nanogels, the usual reactive groups can be thiol or amine groups of the amino acids. Amine based conjugation may exhibit challenges due to abundance of amine groups on the protein and peptide groups. Thus, amine based conjugation may lead complications as conjugation to redundant amines or undesired crosslinking. Since thiol groups of cysteines are more specific targets and unlikely to cause complications during bioconjugations, thiol groups are better candidates for facile bioconjugation [47]. Conjugation of maleimide to thiol is a promising candidate in the field of bioorthogonal chemistry. This reaction can occur even in mild and very dilute conditions with excellent yield and no byproduct [48-52]. Nevertheless, in order to have maleimide groups in polymeric materials, they have to be protected before polymerization due to their susceptibility to attack of free radicals. Diels-Alder (DA) chemistry of furan and maleimide groups were previously used to protect maleimide groups which can be reactivated after the polymerization [53]. To synthesize thiol reactive hydrophilic copolymer, PEG methacrylate monomer and a furan protected maleimide containing methacrylate monomer were used via ATRP. After obtaining the Poly(PEGMEMA-co-FuMaMA), its maleimide groups were activated via retro Diels –Alder reaction (Figure 3.1).

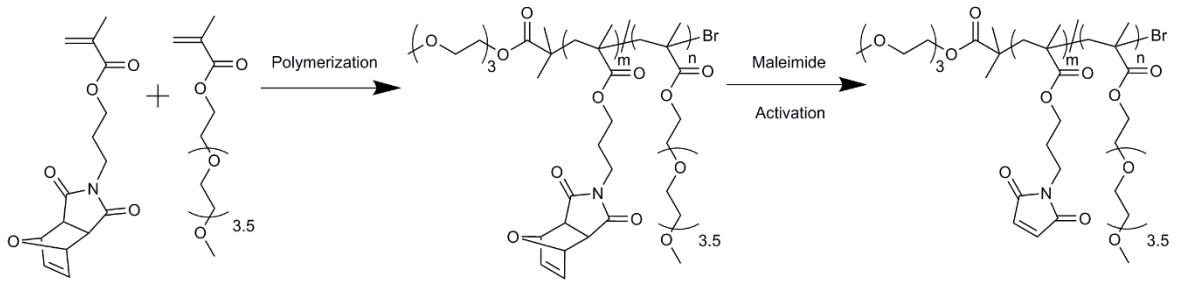

Figure 3.1. Preparation of Thiol Reactive Polymer

3.1.1. Synthesis of Copolymers Bearing Maleimide Side – Chains

The furan protected maleimide containing monomer was used to synthesize the masked maleimide group containing monomer as in figure 3.2. [54]. The monomer was synthesized according to a procedure developed in our laboratory [55]. Firstly, Diels-Alder reaction was performed between furan and maleic anhydride. After that 3-aminopropanol was added to the cycloadduct to obtain a furan protected maleimide group. Then methacryloyl anhydride was added to second product to obtain a furan protected maleimide bearing monomer. (Figure 3.2.)

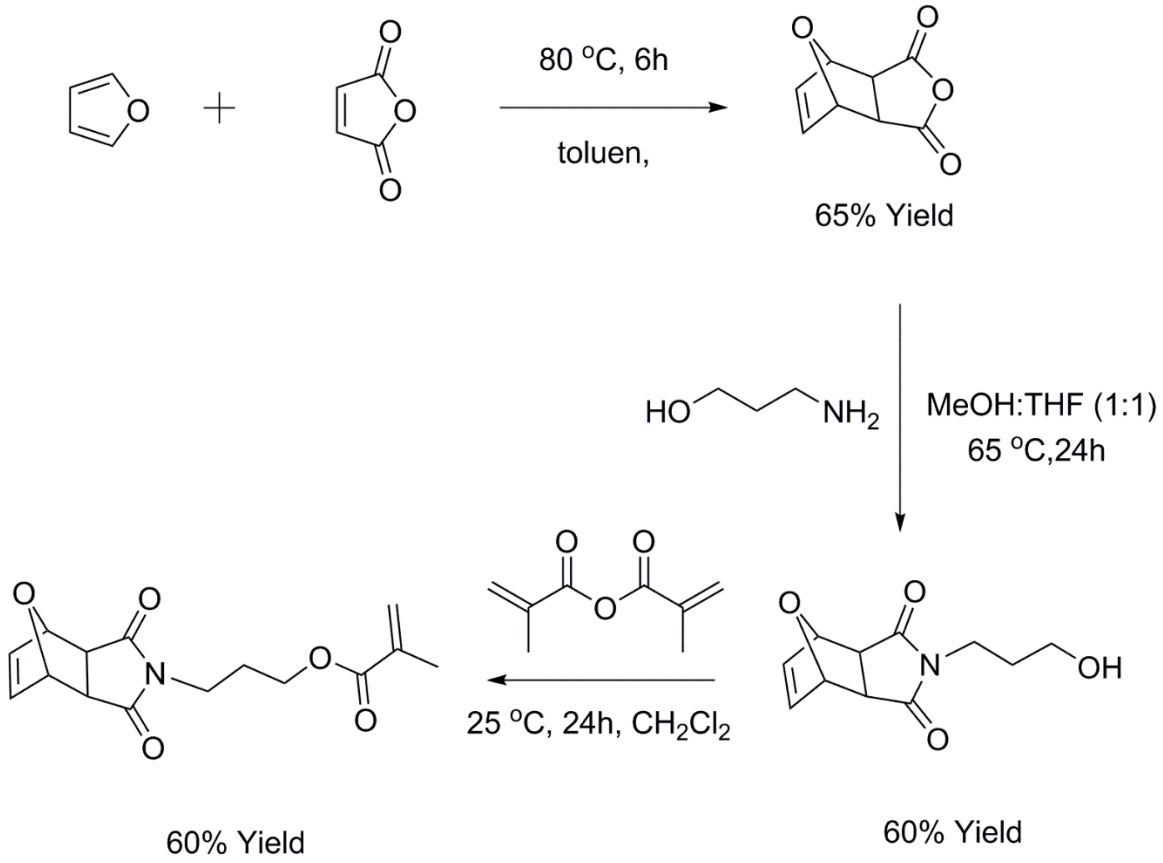

Figure 3.2. Synthesis of FuMaMA monomer

The masked maleimide containing monomer was polymerized with the commercially available poly(ethylene glycol)monomethylether methacrylate (PEGMEMA, M_n = 300 g mol^{-1}) at room temperature via ATRP according to previously reported procedure from our group (P1, Figure 3.3)[56]. PEGMEMA monomer was selected in order to have hydrophilic polymers with anti-biofouling and LCST characteristics. ATRP was selected because it can

be conducted at low temperatures and yields high molecular weight polymers in a very short time. Molecular weight and polydispersity of polymer was analyzed via GPC (M_n, 21000; PDI 1.6, Figure 3.4). Poly (PEGMEMA-co-FuMaMA) was synthesized with 5 : 1 feed ratio of PEG units to the maleimide groups. The ratio of incorporation of monomers was deduced via comparing the area of NMR peaks belonging to the double bond protons at 6.53 ppm, and bridgehead protons at 5.25, against methoxy protons of PEG side chain appearing at 3.37 ppm. (Figure 3.5.) Feed ratio of PEG unit to maleimide groups are calculated as 4.6:1.

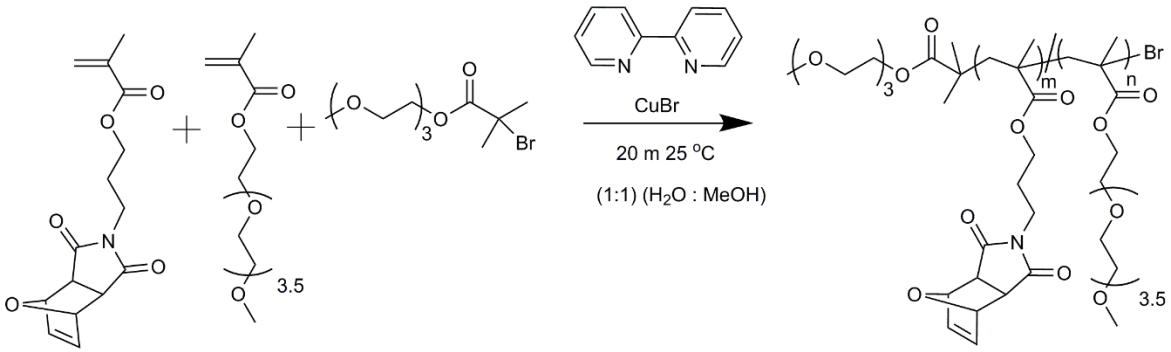

Figure 3.3. Synthesis of P1 via ATRP

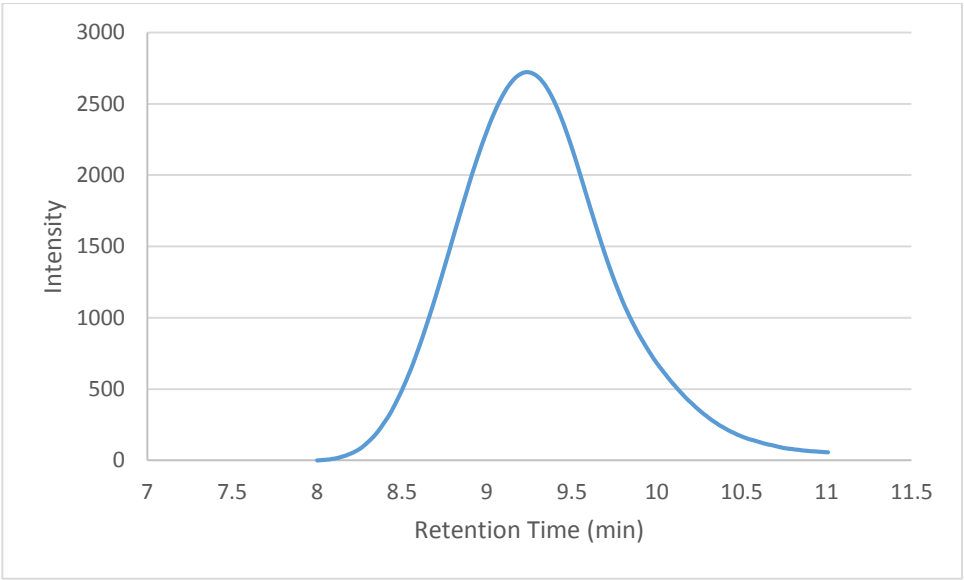

Figure 3.4. Size exclusion chromatogram of P1 analyzed via GPC

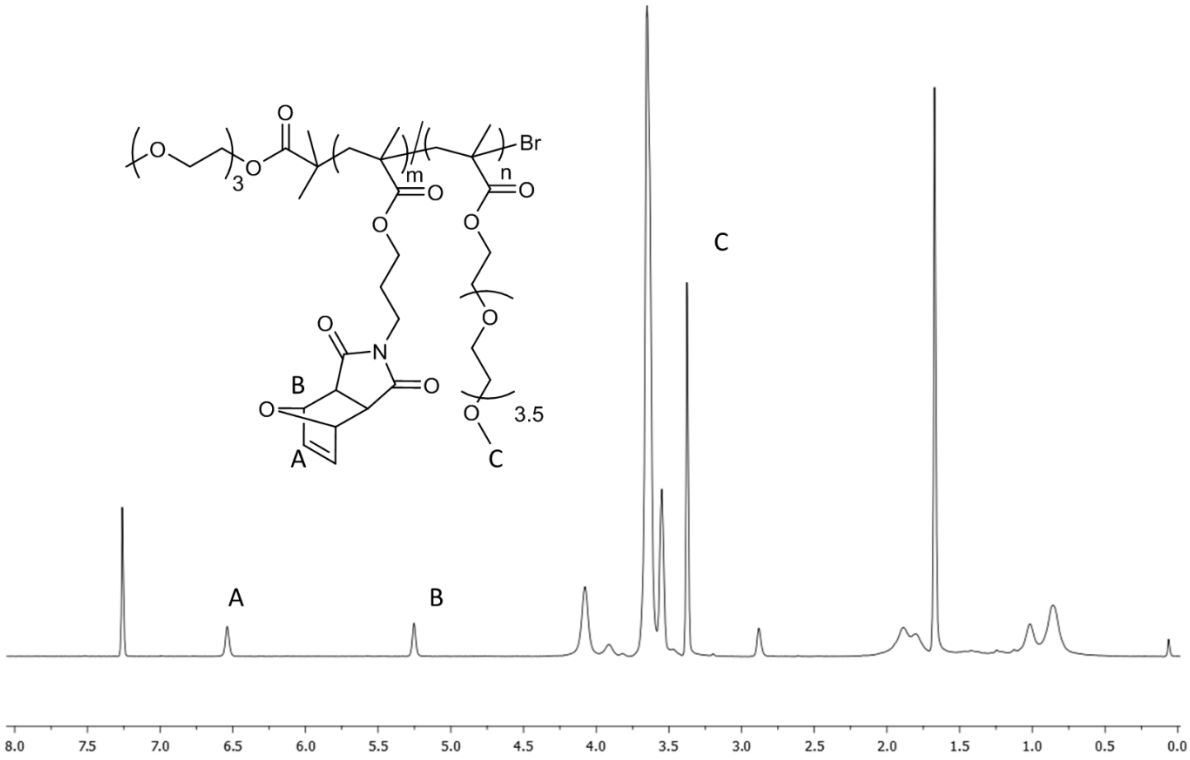

Figure 3.5. NMR Spectrum of P1

3.1.2 Activation of Maleimide Units of poly (PEGMEMA-co-FuMaMA)

After the polymerization maleimide groups were activated into their thiol-reactive forma via retro Diels-Alder reaction by refluxing in toluene at 110 ^{o}C (P2, Figure 3.6).

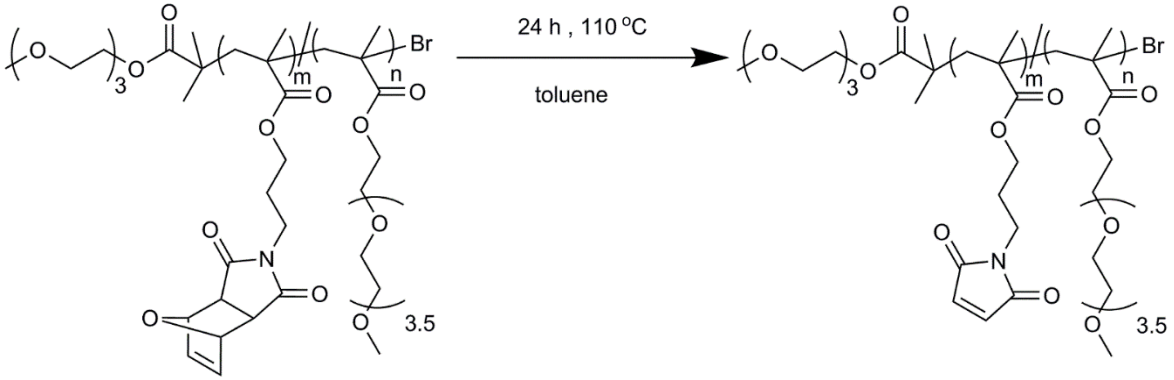

Figure 3.6. Activation of maleimide groups of P1 via retro Diels-Alder reaction

Activation of the maleimide groups were analyzed via 1H NMR spectroscopy. Double bond peak in the spectrum shifted to 6.77 ppm. Feed ratio of PEG units to maleimide moieties were calculated as 4.98:1. As expected, the feed ratio of monomers were mostly preserved and there was little decomposition of the maleimide group and it was verified via comparing the area of 1HNMR peaks belonging to the double bond protons at 6.77 ppm, against methoxy protons at 3.37 ppm. (Figure 3.7.)

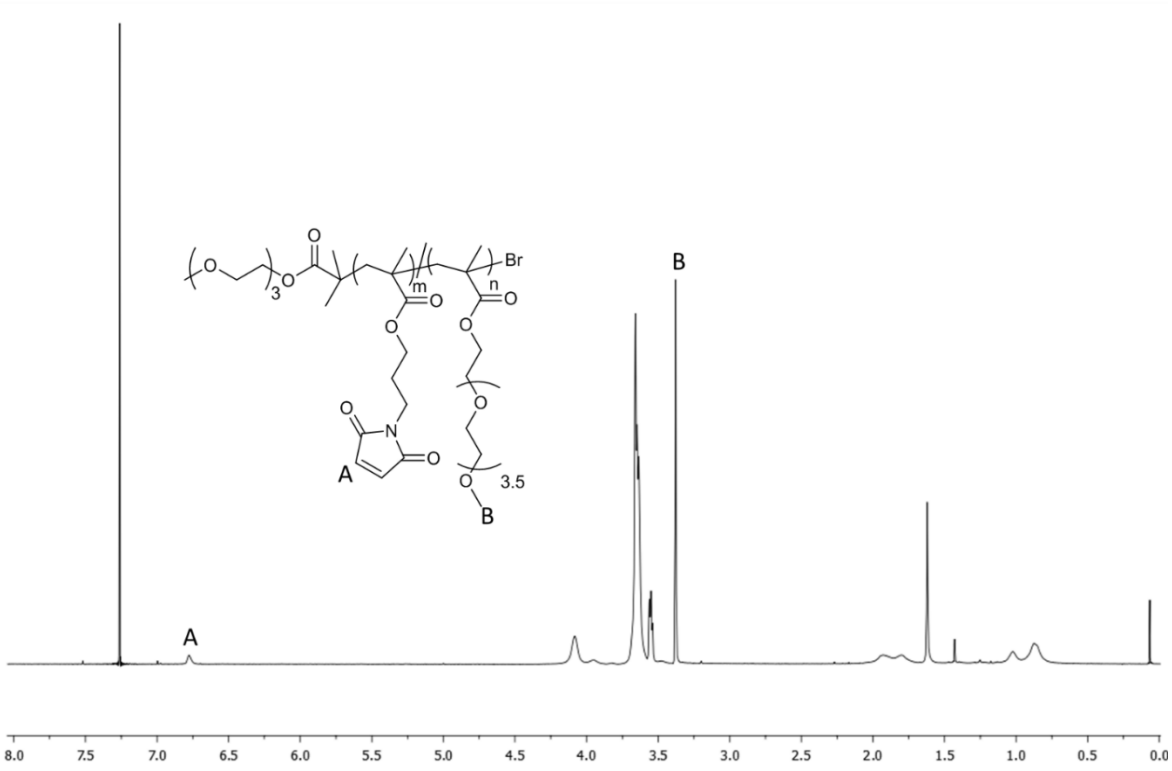

Figure 3.7. NMR spectrum of P2

3.2. Design and Synthesis of Multifunctional Nanogels

As mentioned before, chemically crosslinked nanogels are more desirable than the physically crosslinked ones due to the stability issues. In addition, synthesis of surfactant free nanogels are important since surfactants are difficult to remove and leftover surfactants might have toxic effect on biological systems. Nanogels already have passive targeting ability due to their size and EPR effect. If the nanogels have any clickable group on their surface their selectivity can be improved via addition of targeting groups. In order to achieve all the above mentioned attributes, a methacrylate based copolymer bearing PEGMEMA and maleimide moieties along the side chains was used to obtain a nanogel. This amphiphilic

water soluble copolymer forms stable aggregates above its LCST. Thus, surfactant free nanogels can be synthesized and the size of the self-assembly can be tuned according to temperature or concentration.

3.2.1 Formation of Self–Assembly

In order to form stable self-assembled structures, copolymer P2 has to be heated above its LCST. Size of the self-assembly play the key role in the size of nanogel. Maleimide moieties can be used for crosslinking with the addition of a dithiol bearing crosslinker. In addition both leftover maleimides and thiols on the surface can be modified after the gelation. Prior to gel formation, P2 was heated the in aqueous solution to 60 ^{o}C to obtain the self-assembly. The size of this aggregate was investigated by using dynamic light scattering (DLS). It was measured as 192 nm at 60 ^{o}C and 7 nm at room temperature (Figure 3.8).

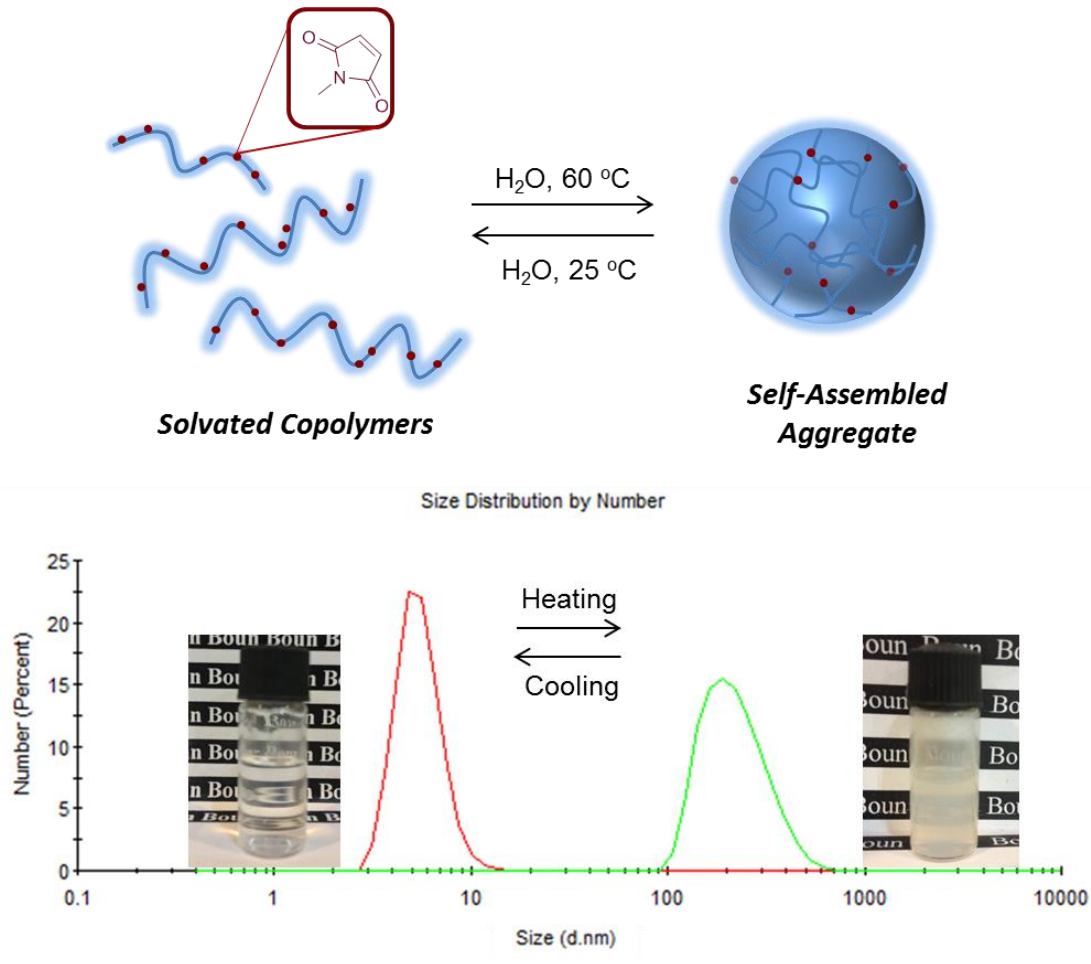

Figure 3.8. Self-Assembly formation of P2 in aqueous solution above its LCST temperature and its size measurements via DLS at 25 and 60 ^{o}C

To determine the LCST temperature, 2 mg/mL aqueous solution of P2 was analyzed using UV spectroscopy. Measurements were made at 600 nm between 5 ^{o}C intervals from 30 to 70^{o}C. LCST of P2 was determined around 55^{o}C according to transmittance change of P2 solution (Figure 3.9).

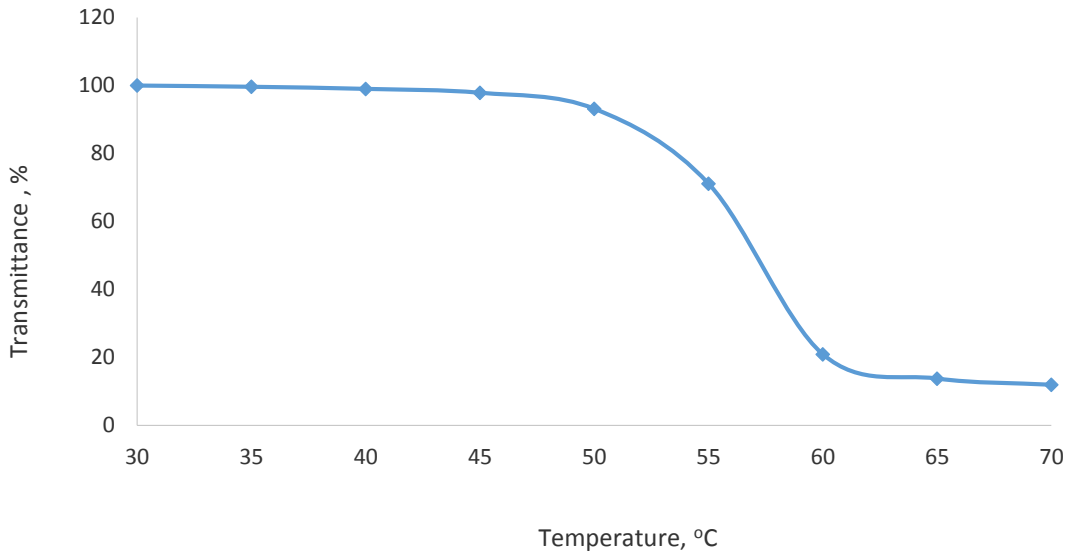

Figure 3.9. Variation in % transmittance of P2 at 600nm with temperature

3.2.2 Synthesis of Multi-clickable Nanogels

To synthesize the nanogel 5 mg of polymer is dissolved in 1.5 mL dH_2O. After formation of the polymeric self-assembly, half mole percent of 2,2'-(ethylenedioxy)-diethanethiol was added against total maleimide units of the polymer as in Figure 3.9.

Nanogels were characterized by using DLS and transmission electron microscopy (TEM). Hydrodynamic diameter of the nanogel solution is found as 88 nm via DLS and 80 nm via TEM (Figure 3.10). This size difference is expected due to swelling of the gels in the solution. Normally, the aqueous polymer solution was clear at room temperature. The solution became turbid and white colored upon heating to 60 ^{o}C. This self-assembly and turbidity formation was reversible for polymer, however solution maintained its turbidity at room temperature after gelation reaction.

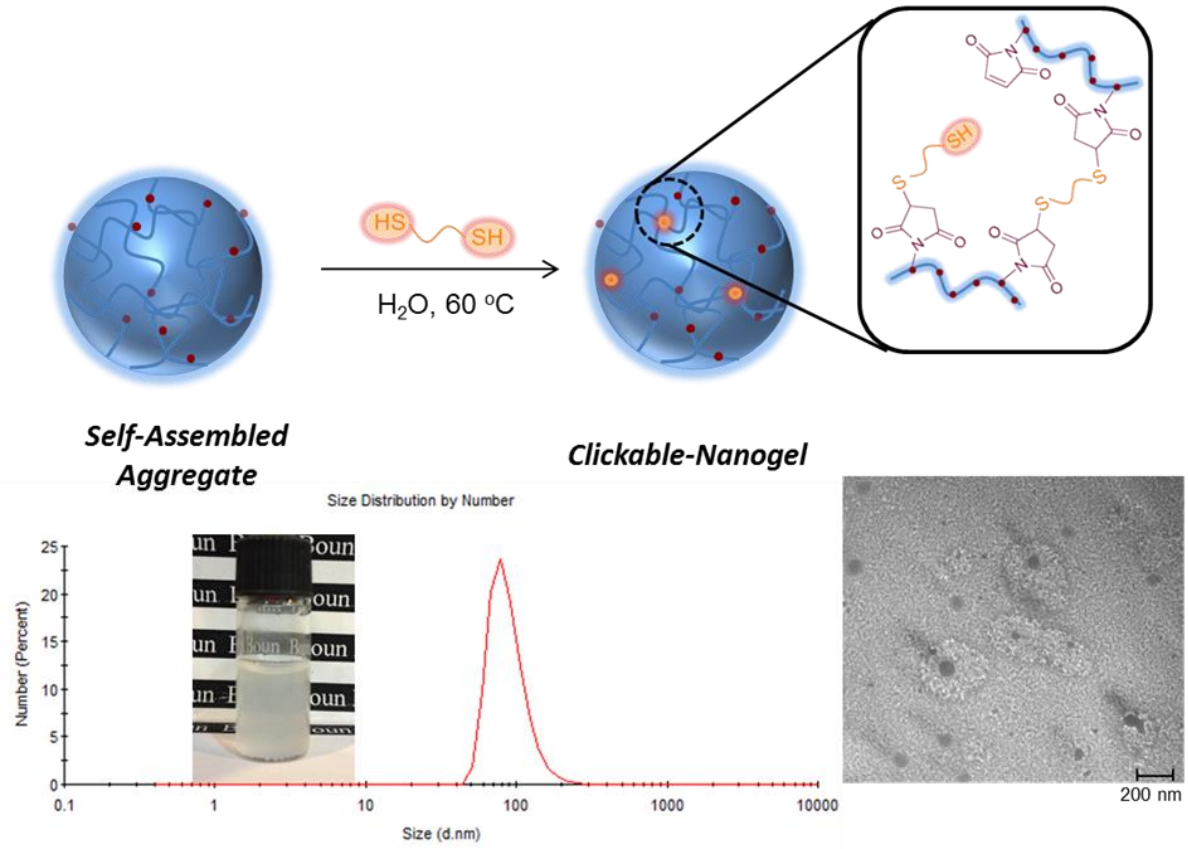

Figure 3.10. Schematic presentation of gelation reaction of P2, nanogel size at 25 °C and TEM image

3.2.3. Effect of Concentration and Temperature on Size of Nanogels

To investigate the effect of concentration on the size and yield of nanogels, 5 mg of P2 was dissolved in the 1.0, 1.5, 2.0, 3.0 mL dH_2O respectively. The size of the nanogels were measured using DLS. (Samples were filtrated using 225nm Cellulose Acetate membrane.) Nanogel synthesized with 5.0, 3.3, 2.5 mg/mL polymer had 111, 88, 76 nm size respectively (NG1, NG2, NG2, Figure 3.11). Nanogels were filtrated prior to DLS analysis. The yields of gelation reactions were 40%. The P2 solution of 1.6 mg/mL yield no nanogel.

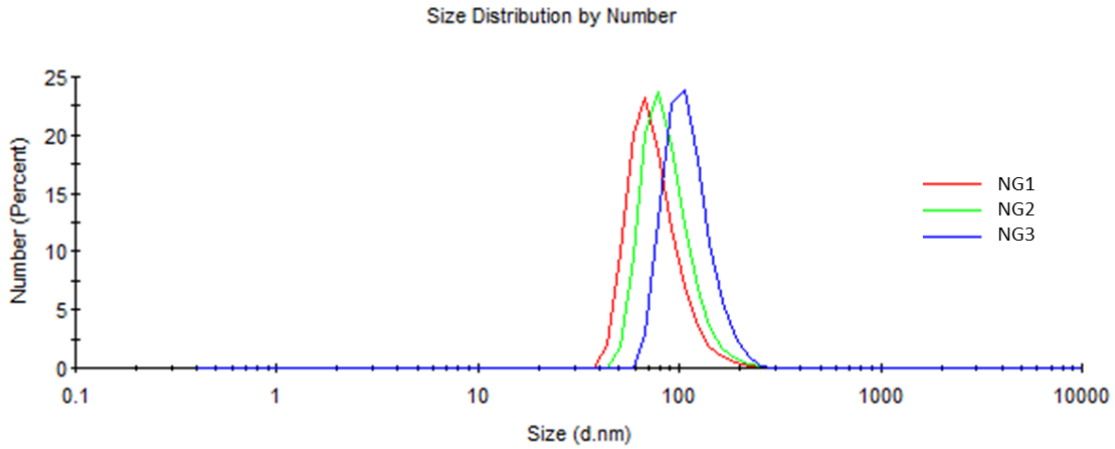

Figure 3.11. Sizes of the nanogels prepared with different P2 concentrations

To investigate the effect of temperature on the size and yield of nanogels, 3.3 mg/mL polymer reaction was run at 60, 65 and 70 ^{o}C (NG2, NG4, NG5). No significant size increase of nanogels was observed according to temperature. However, 4.5 % of the formed nanogels at 65 and 70 ^{o}C nanogels had sizes around 500-600nm as in Figure 3.12. However, the yield was 52 % and 90% for the nanogels synthesized at 65 and 70 ^{o}C. As expected, increase of the temperature increased the speed of reaction however, it disrupted the size control and caused larger aggregates.

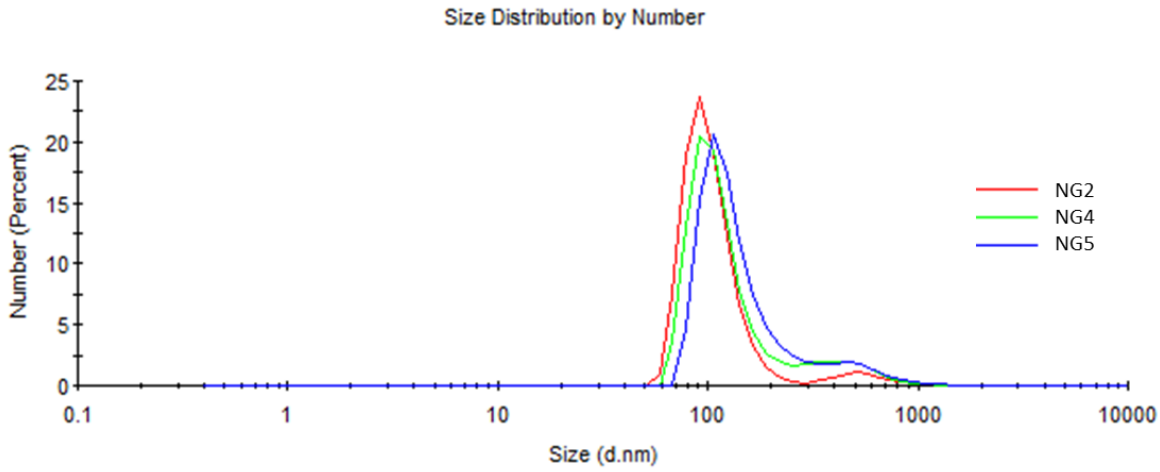

Figure 3.12. Sizes of the nanogels prepared at different temperatures

3.3. Synthesis BODIPY Dyes

BODIPY is a hydrophobic dye which contains a dipyromethene complex with a disubstituted boron. A BODIPY based dye with bromine end group and a new BODIPY based thiol bearing dye is synthesized for the conjugation to the maleimide groups of the nanogels.

3.3.1 Synthesis of BODIPY-SH via BODIPY-Br

BODIPY-Br was synthesized according to literature [57] . For the synthesis of BODIPY-SH, BODIPY-Br was used as in the figure 3.13. Bromine group of BODIPY-Br was attacked by a thio acetate base. After that the acetate groups was hydrolyzed.

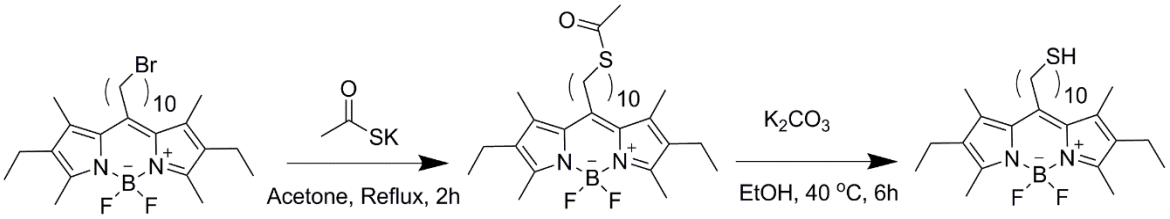

Figure 3.13. Synthesis of BODIPY-SH via BODIPY-Br

3.4. Characterization of Multi-clickable Nanogels

To characterize the nanogels, different dyes with thiol or maleimide groups were conjugated to nanogels. In addition, P2 polymer and NG2 were compared using FTIR spectroscopy. Lastly, a targeting group, cyclic peptide, was conjugated to nanogels.

3.4.1 Conjugation of BODIPY Dyes to Nanogel

Conjugation of the dye, BODIPY-SH, to the nanogels were carried out in the THF since the dye is insoluble in the water (Figure 3.14). To help the Michael addition reaction trimethylamine was used as a catalyst.

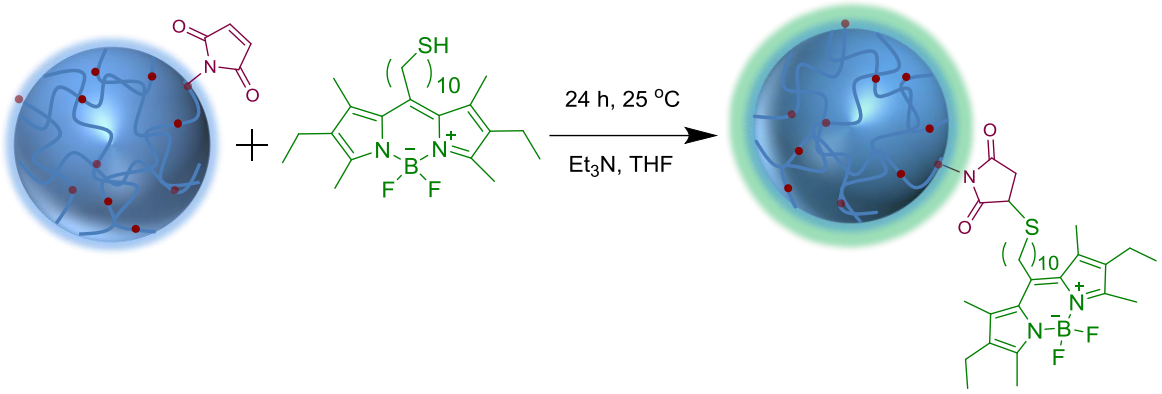

Figure 3.14. Conjugation of BODIPY-SH to NG2

In addition, to prove dye was not incorporated in the core of the nanogel but chemically bound to maleimide another BODIPY dye (BODIPY-Br) is used for conjugation. After the reaction of the nanogel with both BODIPY–SH and BODIPY–Br, nanogels were dispersed in water again. When two samples were exposed to UV light, there was no fluorescence emission from the nanogel treated with BODIPY –Br however the nanogel treated with BODIPY–SH (NG6) showed fluorescence (Figure 3.15)

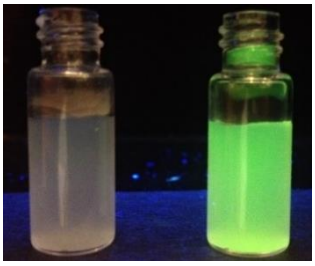

Figure 3.15. Photograph of UV-illuminated aqueous solutions of NG2 after treated with BODIPY-Br (Left) BODIPY-SH (Right)

In addition, the fluorescence of the nanogels were observed via fluorescence spectroscopy as shown in figure 3.16. While strong fluorescence was observed for dyes chemically conjugated to the nanogels, very little fluorescence was observed for nanogels treated with a BODIPY-Br dye that did not possess any thiol group. The experiments proved the thiol–maleimide conjugation of dye and nanogel and there was no physical encapsulation of the dye. The fluorescence emerged from the dye enables the nanogel to be used as an imaging agent in aqueous solutions.

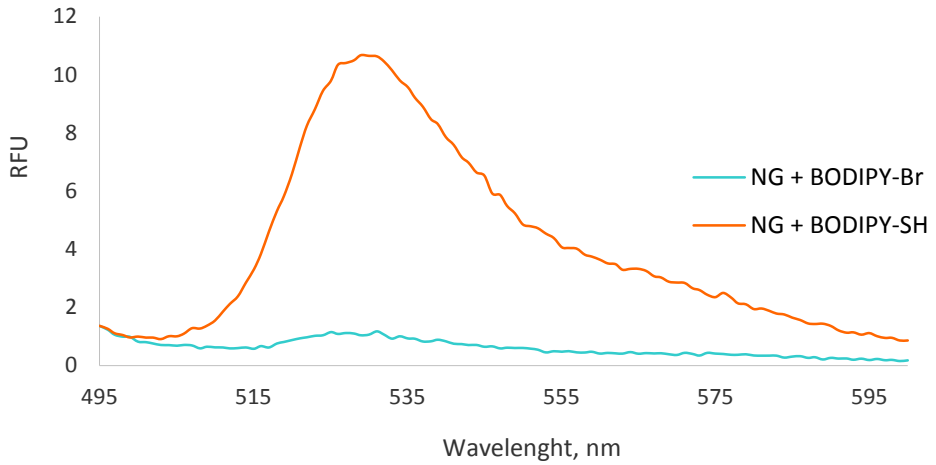

Figure 3.16. Fluorescence spectra of nanogels reacted with BODIPY-Br and BODIPY-SH

3.4.2 Conjugation of cRGDfC and N-(Fluoresceinyl) Maleimide to Nanogel

To show the multifunctional nature of the nanogels, their maleimide groups were conjugated with a cyclic peptide consisting of five amino acids carrying a free thiol, cRGDfC (NG7, Figure 3.17).

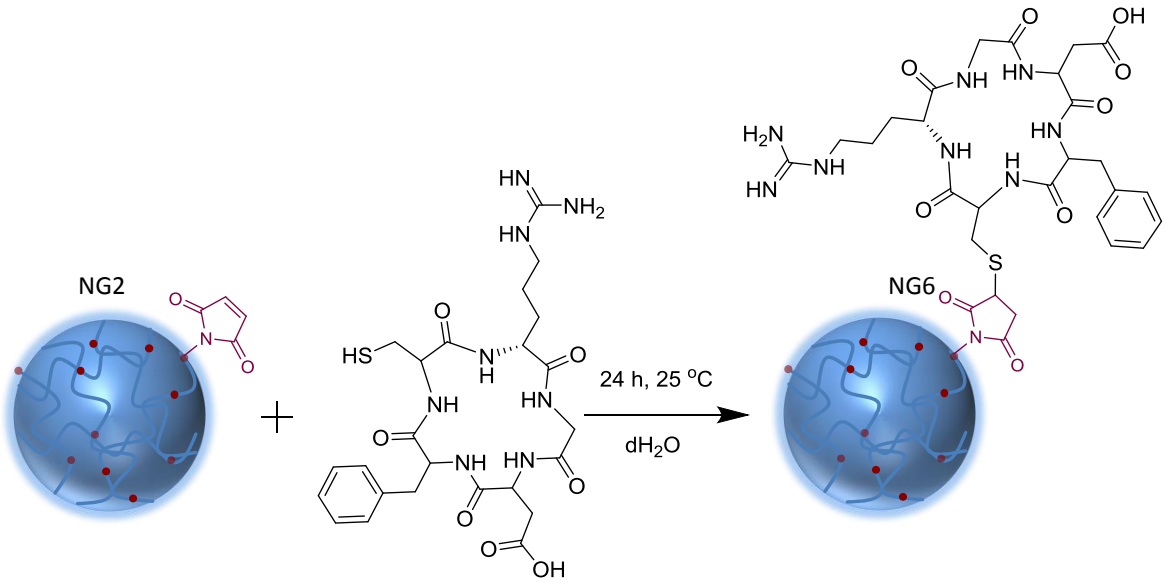

Figure 3.17. Conjugation of cRGDfC to NG7

Due to the hydrophilic nature of the cRGDfC, the reaction was conducted in aqueous

media at room temperature. The leftover unreacted peptide was removed via dialysis. In order to calculate the total amount of conjugated cRGDfC, bicinchoninic acid (BCA Assay) was used. The nanogel samples were incubated in BCA working reagent for 1h at 60 °C. After incubation absorbance of the solution was measured in UV spectrometry at 562 nm. Calibration curve of BSA standard was used to calculate the amount of cRGDfC in nanogels. Weight % of cRGDfC in nanogels calculated as 9%. (0.16 µmol cRGDfC in 1mg NG).

After that reaction thiol groups of the NG6 were conjugated with N-(fluoresceinyl) maleimide (NG8, Figure 3.18). Concentration of the leftover dye is calculated using a UV calibration curve at 480 nm. Weight % of N-(fluoresceinyl) maleimide in nanogels calculated as 1.25%. (0.026 µmol dye in 1mg NG).

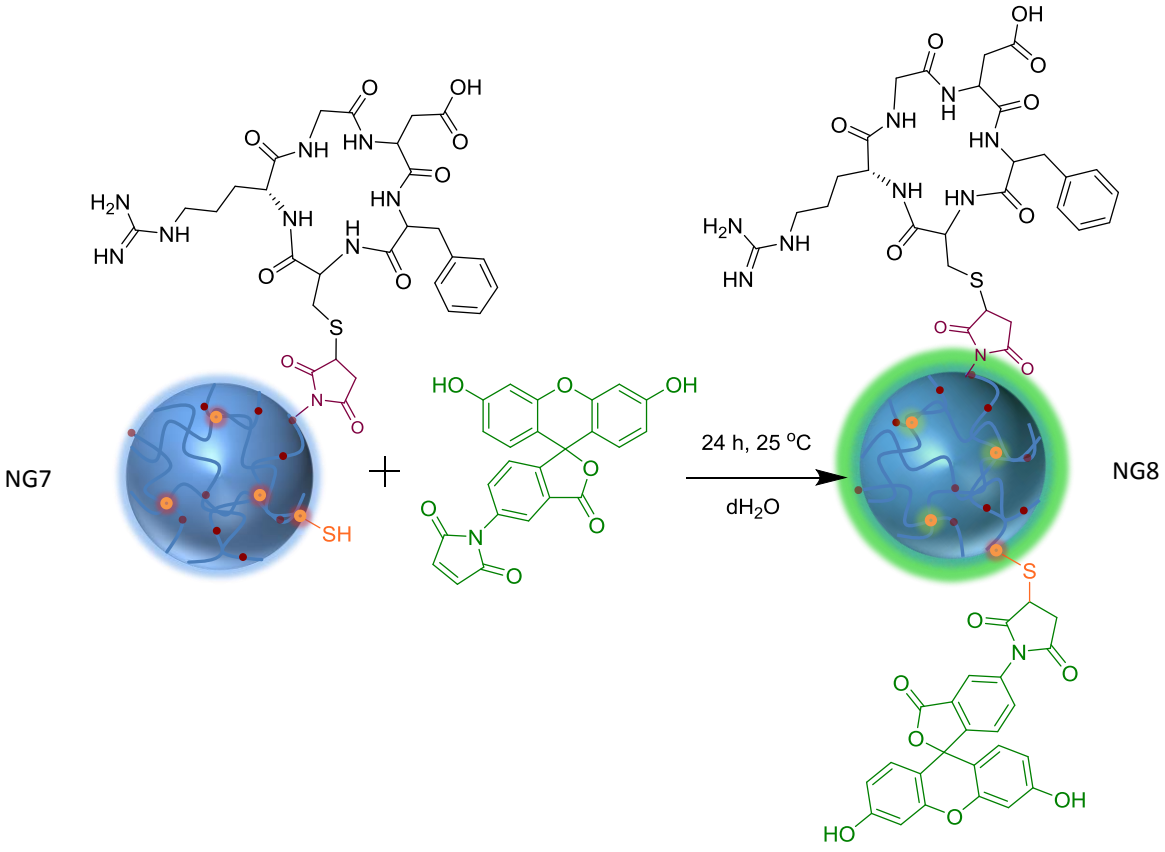

Figure 3.18. Conjugation of N-(Fluoresceinyl) maleimide to NG8

To functionalize the residual free thiols on the nanogels, a maleimide bearing hydrophilic fluorescent dye, N-(fluoresceinyl) maleimide was used.. It can be conjugated to free thiol groups of the nanogels by using its maleimide units via Michael addition. Free

thiol groups on the nanogel depend on the molar ratio of P2 over crosslinker. When the ratio of crosslinker over P2 is increased it is expected to have more thiol groups in the nanogel. To investigate this phenomena, three nanogels with different maleimide units of P2 to thiol groups of crosslinker ratio were synthesized; 100%, 50% and 25% respectively (NG9, NG10, NG11). Then, polymers were treated with N-(fluoresceinyl) maleimide. After the reaction yellow powder-like nanogels were obtained which were white prior the addition of the dye. The change of UV peak at 480nm via UV spectroscopy was measured. It was observed that the UV absorbance at 480nm increased upon increase of crosslinker ratio to P2 (Figure 3.19). Therefore, it is concluded that the amount of maleimide and thiols can be changed by altering crosslinker ratio without changing the size of the nanogels. Moreover, as a control experiment, we treated NG2 with ethyl maleimide (NG12). After treating the NG11 with N-(fluoresceinyl) maleimide, no color change was observed and no peak at 480nm appeared. Therefore, it proves that the dye is not physically encapsulated but it is conjugated to nanogel via the thiol–maleimide addition.

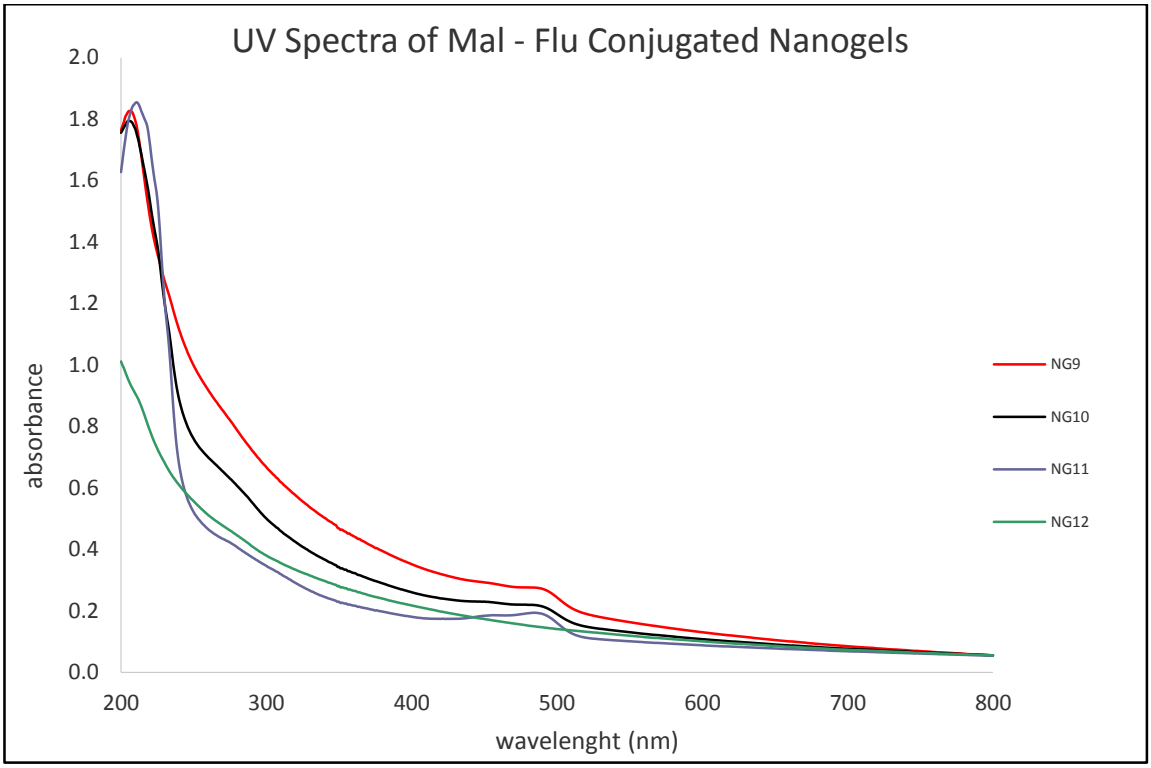

Figure 3.19. UV Spectra of N-(fluoresceinyl) maleimide treated nanogels

FTIR spectra of P2 and NG2 were compared. The peak at 1725 cm^{-1} was from the carbonyl group of the ester, whereas the peak at the 1703 cm^{-1} was due to the imide of the

maleimide ring (Figure 3.20.). Both P2 and NG2 possesed these peaks. Thus, it was concluded that no hydrolysis of maleimide ring occurred during gelation.

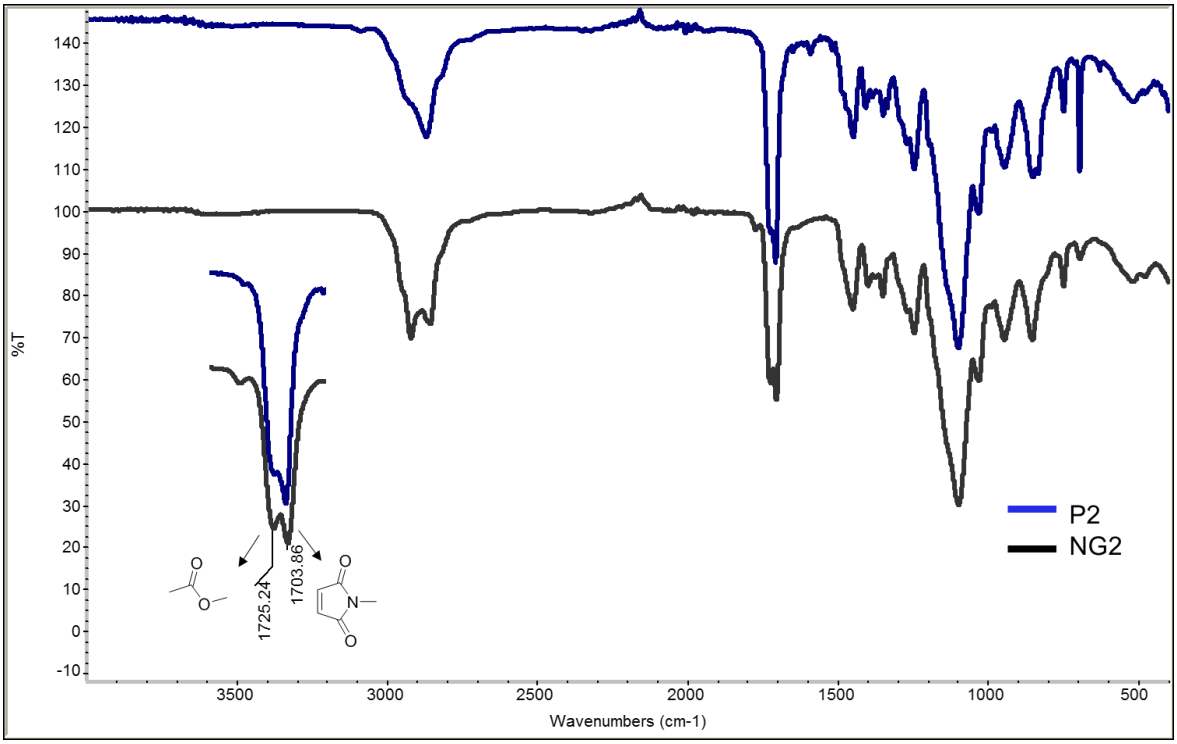

Figure 3.20. FTIR Spectra of P2 and NG2

3.5. In Vitro Cytotoxicity Assay

To use the nanogels as imaging agents they should not possess cytotoxicity. To investigate this effect, MDA-MB-231, human breast adenocarcinoma cells, were treated with nanogels. Cells were incubated in 96-well plates and treated with nanogels, NG2, for 48h at 37 ^{o}C. CCK-8, a colorimetric assay, was performed to distinguish cell viability. Absorbance values at 450 nm were measured by GraphPad prism software which uses non-linear regression curve with a sigmodial dose response equation. The nanogels did not show any cytotoxicity even at highest concentration as in Figure 3.18. Thus, it can be concluded that the EC_{50} value, the concentration at which the toxicity of the construct causing toxicity over the half of the cells, should be higher than 1 mg/ml. The nontoxic behavior of the

nanogels have indicated the potential of nanogels as a promising imaging agent.

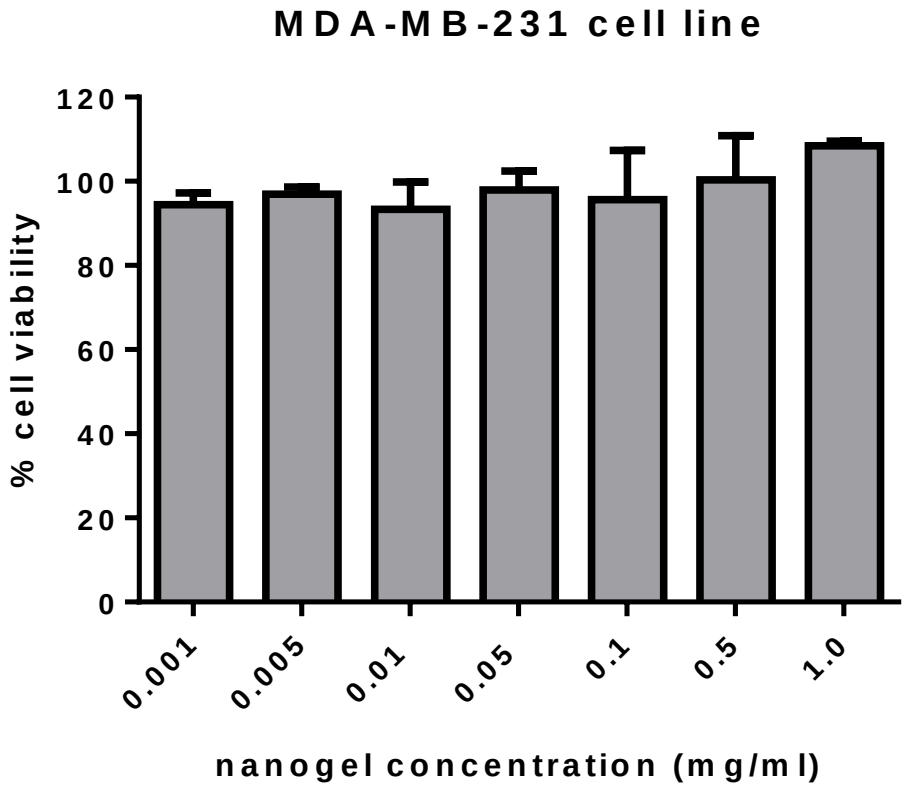

Figure 3.21. Cytotoxicity assay of NG2 on MDA-MB-231 cells. Cell viability was determined via CCK-8 assay

3.6. Cellular Internalization

To investigate the cellular uptake of the fluorescein conjugated nanogels, adenocarcinoma MDA-MB-231 human breast cell line were treated with the nanogels. Green fluorescence ability of the fluorescein enabled the visualization of the cells. To stain and visualize the nuclei of the cells DAPI, 4',6-diamino-2-phenylindole, was used due to its blue fluorescence. Cultured cells were treated with nanogel samples (50 μg/ml) and incubated at 37 ^{o}C. Two constructs were designed for the experiment; one is conjugated to only fluorescein the other is conjugated to both fluorescein and cRGDfC. The overexpressed integrin proteins on the surface of MDA-MB-231 cells make them a good target for the constructs carrying cRGDfC on their surface. Cellular uptake of the nanogels were screened at time points (3, 6 and 24h) via fluorescence microscopy (Figure 3.22). The cells treated

with nanogels including RGD group have shown higher fluorescence intensity than the cells treated with nanogels lacking RGD group on their surface. The results conclude that the targeting group, cRGDfC, on the nanogels boost their internalization and increase their potential as a selective imaging agent.

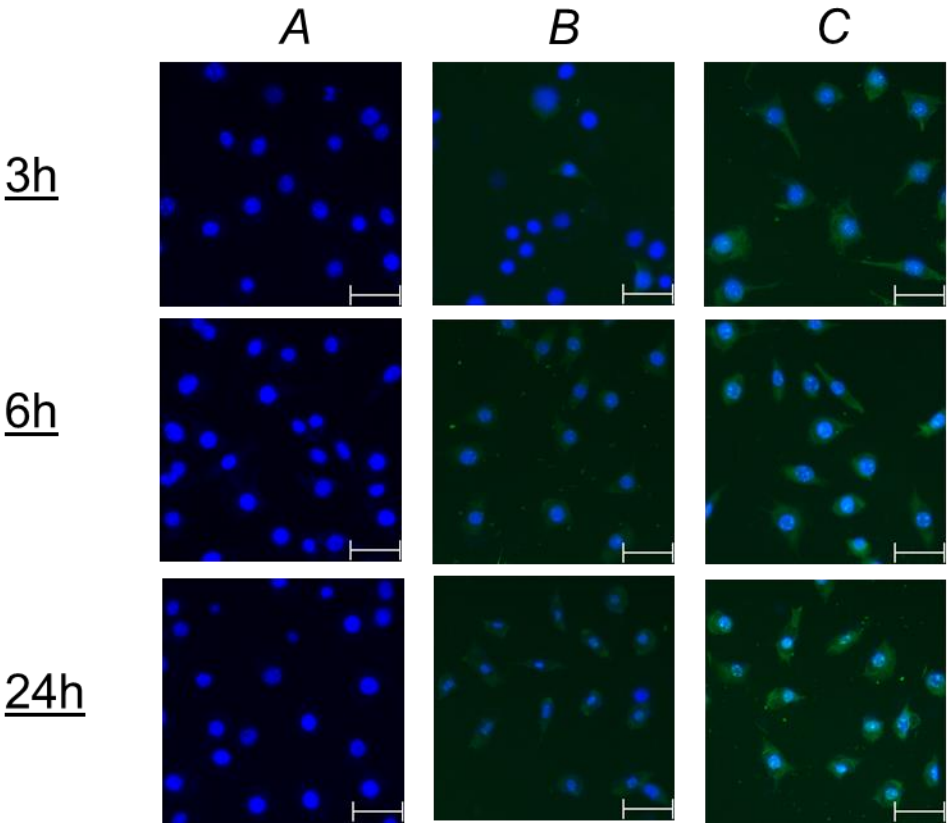

Figure 3.22. Merged fluorescence images of MDA-MB-231 cells are treated as following (A) Control, (B) Nanogels with only Mal - Flu, and (C) Nanogels first conjugated with cRGDfc then Mal- Flu.Cells were incubated at 37 °C for different time points (3, 6, and 24 h). The scale bar is 50 μm.

3.7. Quantification of Internalized Dye via Flow Cytometry

In order to quantify and compare the amount of internalized fluorescent dye in the MDA-MB- 231cells, flow cytometry experiment was performed. For the experiment, the nanogel conjugated with only N-(Fluoresceinyl) maleimide (NG10) and nanogels conjugated with both N-(Fluoresceinyl) maleimide and cRGDfC (NG8) were used. After the incubation of the cells with 0.1 mg/mL nanogel constucts, cells were trypsinized and

centrifuged at 3h, 6h and 24h. Change of media enable the removal of free nanogels and by using flow cytometry only internalized nanogels were quantified.

The flow cytometry results show that in 3h, amount of fluorescence in cells were similar to internalization experiment. Nanogels having cRGD group (blue histogram) showed more fluorescence than the nanogels without cRGD group (red histogram) in 3h as in Figure 3.23.

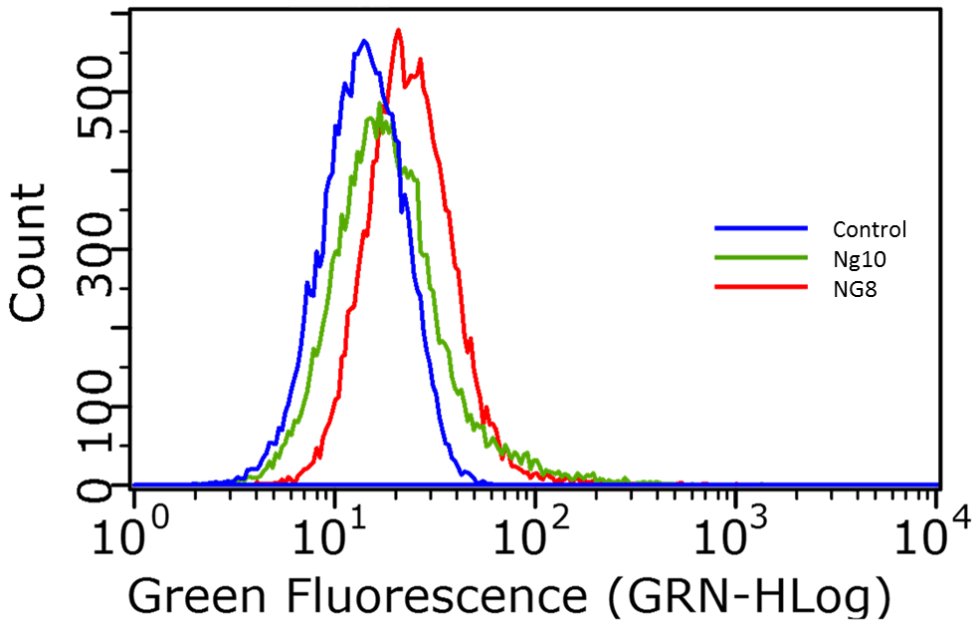

Figure 3.23. Flow cytometry histogram data of NG8 and NG10 on MDA-MB-231 cells at 3h

In addition the fluorescence intensity of the cells, increased at 6h and nanogels bearing cRGD group (blue histogram) showed greater fluorescence intensity than the nanogels without cRGD group (red histogram) as in Figure 3.24. Yellow histograms were control group cells which were incubated with no nanogel. Since intensity of the cells treated with NG8 are higher than cells treated with NG10, positive effect of targeting group in cellular internalization is proved.

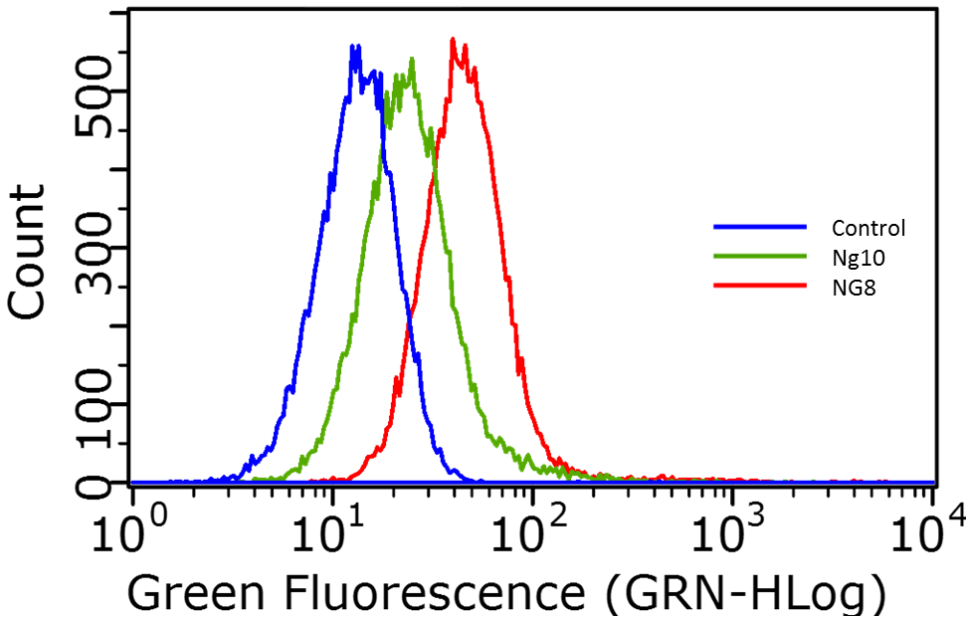

Figure 3.24. Flow cytometry histogram data of NG8 and NG10 on MDA-MB-231 cells at 6h

4. EXPERIMENTAL

4.1. Synthesis of Maleimide Bearing Polymers

To synthesize the copolymer, the furan protected monomer (FuMaMA) (0.2 g, 0.69 mmol) and PEGMEMA (1.00 mL, 3.47 mmol) were dissolved in 3 mL degassed MeOH. The monomer solution was added into the flask containing CuBr (5.1 mg, 0.041 mmol) and 2,2' bipyridine (12.7 mg, 0.082 mmol) in 3 mL degassed H_2O. TEGME-Br (12.6 mg, 0.041 mmol) initiator was introduced into the stirring solution and the reaction was stirred at 25 ^oC for 15 min. Reaction was finished upon opening the flask to the atmosphere. The MeOH was evaporated under vacuum whereas lyophilizer was used for the removal of H_2O. The polymer was dissolved in minimum amount of THF and precipitated in cold diethyl ether. After precipitation, the remaining copper complex is removed via passing the polymer through an aluminum oxide column. After purifications polymer was obtained as colorless and viscous solid. ($[M_0]/ [I_0]$ = 100, $[FuMaMa_0]$: $[PEGMEMA_0]$ = 1:5, $[I_0]$: [CuBr]: [PMDETA] = 1:1:2, conversion 75% $M_{n,theo}$ = 30135 $M_{n,GPC}$ = 21kD, M_w/M_n =1.54 relative to PS. ([FuMaMa]:[PEGMEMA] = 1:4.6) ^1H NMR, ($CDCl_3$, δ, ppm) 6.53 (s, 2H, CH=CH), 5.25 (s, 2H, CH bridgehead protons), 4.07 (s, 2H, OCH_2 ester protons of PEGMEMA), 3.91 (br s, 2H, OCH_2), 3.65 – 3.54 (m, 4H, OCH_2 of PEGMEMA an NCH_2), 3.37 (s, 3H, OCH_3 of PEGMEMA), 2.88 (s, 2H, CH-CH, bridge protons), 1.88 – 0.85 (m, 7H, $NCH_2CH_2CH_2O$, CH_2 and CH_3 protons along polymer backbone).

4.2. Activation of Maleimide Groups of Copolymers via Retro Diels/Alder Reaction

The polymer (300 mg) is dissolved in anhydrous toluene and heated for 24h at 110 ^oC. (380 mg, 96% yield) Degradation of oxabicyclic group and activation of maleimide groups was analyzed by ^1H NMR anaylsis. ([FuMaMa]:[PEGMEMA] = 1:4.98) ^1H NMR, ($CDCl_3$, δ, ppm) 6.77 (s, 2H, CH=CH), 4.07 (s, 2H, OCH_2 ester protons of PEGMEMA), 3.94 (br s, 2H, OCH_2), 3.65 – 3.54 (m, 4H, OCH_2 of PEGMEMA an NCH_2), 3.37 (s, 3H, OCH_3 of PEGMEMA), 1.91 – 0.86 (m, 7H, $NCH_2CH_2CH_2O$, CH_2 and CH_3 protons along polymer backbone).

4.3. Synthesis of Nanogel

Nanogel with 3 different polymer over crosslinker ratio were synthesized. In each 3 flasks, 5 mg polymer was dissolved in 1.3 mL water and heated at 60 ^{o}C for 10minutes. After 10 minutes, 0.1 mL of 16.6 mM, 8.3 mM, 4.2 mM 2,2'-(Ethylenedioxy)-diethanethiol were added into polymer solutions respectively. After addition of crosslinker 0.1 mL of 4.2, mM, 2.1 mM, 1.1 Mm Et_3N were added into polymer solutions respectively and the reactions were stirred at 60 ^{o}C for 1h. The nanogels were centrifuged at 7000 rpm for 25 min to remove the leftover polymer and catalyst. (2 mg, 40% yield)

4.4. Synthesis of BODIPY-SH and BODIPY-Br

BODIPY-Br was synthesized according to literature [57]. For the synthesis of BODIPY-SH, BODIPY-Br was used. BODIPY-Br (100mg, 0.204 mmol) was added into potassium thioacetate (28 mg, 0.236mmol) in 5mL and stirred under reflux for 2 h. After that, acetone was evaporated and the crude was dissolved in 150 mL DCM and washed with water. It was further purified by silica column chromatography using hexane: ethyl acetate (90:10 v/v) as the eluent. (86 mg, 0.176 mmol, 86% yield) The BODIPY - thioacetate (50 mg, 0.104 mmol), was added to potassium carbonate (43mg, 0.312 mmol) and stirred in ethanol for 6 hour at 40 ^{o}C under N_2 atmosphere and protected from light. It was then washed with NH_4Cl and extracted with DCM. It was further purified using silica gel column chromatography using hexane:ethyl acetate (90:10 v/v) as the eluent. (50 mg, 91% yield)

4.5. Conjugation of Nanogel to BODIPY

For the conjugation of the BODIPY – SH to the nanogel, nanogels were precipitated via centrifuge and dispersed in THF (1 mg/mL). BODIPY-Br (0.11 mg, 0.22 μmol) was added into a nanogel solution (1 mg/mL) and the solution was stirred at room temperature for 24h. BODIPY-SH (0.1 mg, 0.22 μmol) and Et_3N (0.22 μmol) was added into another nanogel solution (1 mg/mL) and the solution was stirred at room temperature for 24h. After both of the reactions the nanogels were centrifuged at 7000rpm for 25 min and were dispersed into water. Nanogel solutions (1 mg/mL) were exposed to UV light at 365 nm. A

handheld UV lamp was used for UV light. (Blak-Ray UVP model B-100AP/R High Intensity UV lamp with a 100-watt spot bulb and 7° beam width). They were 80X diluted and their fluorescence spectra were analyzed.

4.6. Conjugation of Nanogel to Ethyl Maleimide

The nanogel (1 mg/mL) was dispersed in THF. After that ethyl maleimide (125 μg, 1 μmol) was introduced into the nanogel solution and the reaction was stirred for 24 h at room temperature. For the removal of leftover ethyl maleimide, nanogels were centrifuged at 7000 rpm for 25 min and dispersed in water.

4.7. Conjugation of cRGDfC to Nanogel

To the 1 mL nanogel solution (2 mg/mL) in water cRGDfC (250 μm, 0.430 μmol) was added and the reaction was stirred for 24h at room temperature. For the removal of the leftover peptide centrifuge and dialysis was used. After purification of the nanogel 100 μL of the sample was mixed with 2mL BCA working reagent. Then the blue mixture is incubated for 1h at 60 ºC. After 1h the blue mixture turned into purple and its absorbance was measured at 562 nm via UV spectroscopy.

4.8. In Vitro Cytotoxicity

Cytotoxicity of the nanogel is investigated via CCK-8 viabiltiy assay on MDA-MB-231 adenocarcinoma cells. Cells (5000 cells/well) were seeded on the 96-well plate as triplicates in 100 μL culture medium and incubated at 37 ºC for 24 h for cells to grow and adhere completely. Nanogels with 7 different concentrations (1.0, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001 mg/mL) were prepared in PBS (ph 7.4). Nanogel samples were added on the cell media and the cells were incubated at 37 ºC for 48 h. After 48 h solutions were removed and the cells were washed with 100 μL PBS twice. To measure the cell viability CCK-8 was used. 6 μL CCK-8 reagent were used to form the 60 μL of cell media and the cells were incubated at CCK-8 reagent carrying media for 3 h. After 3h of incubation the absorbance values at 450 nm were measured via microplate reader. Results were obtained by GraphPad prism

software using in nonlinear regression mode.

4.9. Cellular Internalization Assay

For the cellular internalization experiment MDA-MB-231 adenocarcinoma cells (50,000 cells/well) were seeded 12-well plate as triplicate in 1 mL culture media. The cells were incubated at 37 ^{o}C FOR 24 h. One of the plates were treated with nanogel (0.1 mg/mL) conjugated to only N-(Fluoresceinyl) Maleimide whereas the other one is treated with the nanogel (0.1 mg/mL) conjugated to both N-(Fluoresceinyl) Maleimide and cRGDfC. After addition of nanogels cellular media are removed at several time points (3, 6 and 24h) during the incubation. After removal cells were washed with 500 µL PBS and then DAPI (5 mg/mL) containing PBS were added to the plates. Cell were incubated at 37 ^{o}C for 20 min for DAPI to stain nuclei. After DAPI staining cell images were obtained by using Zeiss Observer Z1 via fluorescence microscope.

4.10 Flow Cytometry

For the flow cytometry experiment MDA-MB-231 adenocarcinoma cells (50,000 cells/well) were seeded into 12-well plate as dublicate in 1 mL culture media. The cells were incubated at 37 ^{o}C for 24 h. One of the plates were treated with 0.1 mg/mL NG10 whereas the other one is treated with the 0.1 mg/mL NG8. After 3h, and 6h cell media were removed and the cells were trypsinized with 0.05 % trypsin solution. After trypsin neutralization cells were centrifuged at 300 rpm for 5 min. Then the cells were resuspended in 1X PBS buffer and analyzed via flow cytometry.

5. CONCLUSION

Multifunctional and stable nanogels with well-defined size were synthesized using a copolymer consisting of PEG and maleimide moieties. Due to the PEG chains and maleimide moieties, these amphiphilic polymers have LCST temperature around 55 ^{o}C. Above their LCST they start to form aggregates. Gelation was performed in those aggregates in very dilute conditions. It is shown that size can be tuned via changing the concentration and temperature. PEG groups add anti-biofouling character and water solubility to nanogels. Since the nanogels carry both maleimide and thiol groups they can be multifunctionalized using different groups. Thiol containing targeting group, cRGDfC, and maleimide bearing dye (imaging group) were conjugated to the nanogel. Cytotoxicity experiments showed that the nanogels bear no toxicity to cancerous cell lines. Internalization experiments showed the targeting group improved the cellular internalization of the nanogels as deduced from fluorescence microscopy and flow cytometry. Overall, facile synthesis of both thiol and maleimide reactive nanogels were achieved and it was demonsted that they can be used as an imaging agent via conjugating fluorescent dyes.

REFERENCES

1. Xia, Y., P. Yang, Y. Sun, Y. Wu, B. Mayers, B. Gates, Y. Yin, F. Kim, and H. Yan, "One-Dimensional Nanostructures: Synthesis, Characterization, And Applications.", *Advanced Materials*, Vol. 15, pp. 353–389, 2003.

2. De Jong, W.H., and P.J.A. Borm, "Drug Delivery And Nanoparticles: Applications And Hazards.", *International Journal of Nanomedicine*, Vol. 3, pp. 133–149, 2008.

3. Torchilin, V.P., "Multifunctional Nanocarriers.", *Advanced drug delivery reviews*, Vol. 58, pp. 1532–55, 2006.

4. West, J.L., and N.J. Halas, "Engineered Nanomaterials For Biophotonics Applications: Improving Sensing, Imaging, And Therapeutics.", *Annual review of biomedical engineering*, Vol. 5, pp. 285–292, 2003.

5. Motornov, M., Y. Roiter, I. Tokarev, and S. Minko, "Stimuli-responsive Nanoparticles, Nanogels And Capsules For Integrated Multifunctional Intelligent Systems.", *Progress in Polymer Science*, Vol. 35, pp. 174–211, 2010.

6. Soppimath, K.S., T.M. Aminabhavi, a R. Kulkarni, and W.E. Rudzinski, "Biodegradable Polymeric Nanoparticles As Drug Delivery Devices.", *Journal of controlled release : official journal of the Controlled Release Society*, Vol. 70, pp. 1–20, 2001.

7. Uhrich, K.E., S.M. Cannizzaro, R.S. Langer, and K.M. Shakesheff, "Polymeric Systems For Controlled Drug Release.", *Chemical reviews*, Vol. 99, pp. 3181–98, 1999.

8. Galeska, I., T.-K. Kim, S.D. Patil, U. Bhardwaj, D. Chatttopadhyay, F. Papadimitrakopoulos, and D.J. Burgess, "Controlled Release Of Dexamethasone From PLGA Microspheres Embedded Within Polyacid-containing PVA Hydrogels.", *The AAPS journal*, Vol. 7, pp. E231–40, 2005.

9. van Vlerken, L.E., T.K. Vyas, and M.M. Amiji, "Poly(ethylene Glycol)-modified Nanocarriers For Tumor-targeted And Intracellular Delivery.", *Pharmaceutical research*, Vol. 24, pp. 1405–1414, 2007.

10. Rao, J.P., and K.E. Geckeler, "Polymer Nanoparticles: Preparation Techniques And

Size-control Parameters.", *Progress in Polymer Science*, Vol. 36, pp. 887–913, 2011.

11. Anton, N., J.-P. Benoit, and P. Saulnier, "Design And Production Of Nanoparticles Formulated From Nano-emulsion Templates—A Review.", *Journal of Controlled Release*, Vol. 128, pp. 185–199, 2008.

12. Ganachaud, F., and J.L. Katz, "Nanoparticles And Nanocapsules Created Using The Ouzo Effect: Spontaneous Emulsification As An Alternative To Ultrasonic And High-shear Devices.", *ChemPhysChem*, Vol. 6, pp. 209–216, 2005.

13. Mishra, B., B.B. Patel, and S. Tiwari, "Colloidal Nanocarriers: A Review On Formulation Technology, Types And Applications Toward Targeted Drug Delivery.", *Nanomedicine: Nanotechnology, Biology and Medicine*, Vol. 6, pp. 9–24, 2010.

14. Lee, J., E.C. Cho, and K. Cho, "Incorporation And Release Behavior Of Hydrophobic Drug In Functionalized Poly(d,l-lactide)-block–poly(ethylene Oxide) Micelles.", *Journal of Controlled Release*, Vol. 94, pp. 323–335, 2004.

15. Sasaki, Y., and K. Akiyoshi, "Nanogel Engineering For New Nanobiomaterials: From Chaperoning Engineering To Biomedical Applications.", *Chemical Record*, Vol. 10, pp. 366–376, 2010.

16. Oh, J.K., R. Drumright, D.J. Siegwart, and K. Matyjaszewski, "The Development Of Microgels/nanogels For Drug Delivery Applications.", *Progress in Polymer Science (Oxford)*, Vol. 33, pp. 448–477, 2008.

17. Chacko, R.T., J. Ventura, J. Zhuang, and S. Thayumanavan, "Polymer Nanogels: A Versatile Nanoscopic Drug Delivery Platform.", *Advanced Drug Delivery Reviews*, Vol. 64, pp. 836–851, 2012.

18. Chau, Y., F.E. Tan, and R. Langer, "Synthesis And Characterization Of Dextran-peptide-methotrexate Conjugates For Tumor Targeting Via Mediation By Matrix Metalloproteinase II And Matrix Metalloproteinase IX.", *Bioconjugate Chemistry*, Vol. 15, pp. 931–941, 2004.

19. Wang, Z., C. Tiruppathi, R.D. Minshall, and A.B. Malik, "Size And Dynamics Of Caveolae Studied Using Nanoparticles In Living Endothelial Cells.", *ACS Nano*, Vol. 3, pp. 4110–4116, 2009.

20. Smith, M.H., and L.A. Lyon, “Multifunctional Nanogels For SiRNA Delivery.”, *Accounts of Chemical Research*, Vol. 45, pp. 985–993, 2012.

21. Langer, R., and D. a Tirrell, “Designing Materials For Biology And Medicine.”, *Nature*, Vol. 428, pp. 487–492, 2004.

22. Qiao, Z.Y., F.S. Du, R. Zhang, D.H. Liang, and Z.C. Li, “Biocompatible Thermoresponsive Polymers With Pendent Oligo(ethylene Glycol) Chains And Cyclic Ortho Ester Groups.”, *Macromolecules*, Vol. 43, pp. 6485–6494, 2010.

23. Hellmund, M., H. Zhou, O. Samsonova, P. Welker, T. Kissel, and R. Haag, “Functionalized Polyglycerol Amine Nanogels As Nanocarriers For DNA.”, *Macromolecular Bioscience*, Vol. 14, pp. 1215–1221, 2014.

24. Oh, Jung Kwon, Tang Chuanbing, Gao Haifeng, Tsarevsky, V. Nicolay, Matyjaszewski, K., “Inverse Miniemulsion ATRP: A New Method For Synthesis And Functionalization Of Well-Defined Water-Soluble/Cross-Linked Polymeric Particles.”, *Journal of the American Chemical Society*, Vol. 81, pp. 5578–5584, 2006.

25. Koul, V., R. Mohamed, D. Kuckling, H.J.P. Adler, and V. Choudhary, “Interpenetrating Polymer Network (IPN) Nanogels Based On Gelatin And Poly(acrylic Acid) By Inverse Miniemulsion Technique: Synthesis And Characterization.”, *Colloids and Surfaces B: Biointerfaces*, Vol. 83, pp. 204–213, 2011.

26. J. Groll, S. Singh, K. Albrecht, M.M., “Biocompatible And Degradable Nanogels Via Oxidation Reactions Of Synthetic Thiomers In Inverse Miniemulsion.”, *J. Polym. Sci. Part A: Polym. Chem.*, Vol. 47, pp. 5543–5549, 2009.

27. An, D., D. Zhao, X. Li, X. Lu, G. Qiu, and K.J. Shea, “Synthesis Of Surfactant-free Hydroxypropylcellulose Nanogel And Its Dual-responsive Properties.”, *Carbohydrate Polymers*, Vol. 134, pp. 385–389, 2015.

28. Dimitrov, I., B. Trzebicka, A.H.E. Mülller, A. Dworak, and C.B. Tsvetanov, “Thermosensitive Water-soluble Copolymers With Doubly Responsive Reversibly Interacting Entities.”, *Progress in Polymer Science (Oxford)*, Vol. 32, pp. 1275–1343, 2007.

29. Yallapu, M.M., M. Jaggi, and S.C. Chauhan, “Design And Engineering Of Nanogels For

Cancer Treatment.", *Drug Discovery Today*, Vol. 16, pp. 457–463, 2011.

30. Rzaev, Z.M.O., S. Dinçer, and E. Pişkin, "Functional Copolymers Of N-isopropylacrylamide For Bioengineering Applications.", *Progress in Polymer Science (Oxford)*, Vol. 32, pp. 534–595, 2007.

31. Weber, C., R. Hoogenboom, and U.S. Schubert, "Temperature Responsive Bio-compatible Polymers Based On Poly(ethylene Oxide) And Poly(2-oxazoline)s.", *Progress in Polymer Science*, Vol. 37, pp. 686–714, 2012.

32. Ryu, J.-H., R.T. Chacko, S. Jiwpanich, S. Bickerton, R.P. Babu, and S. Thayumanavan, "Self-Cross-Linked Polymer Nanogels: A Versatile Nanoscopic Drug Delivery Platform Supporting Info.", *Journal of the American Chemical Society*, Vol. 3, pp. 2–10, 2010.

33. Huang, H., K.L. Wooley, H. Huang, and E.E. Remsen, "Amphiphilic Core–shell Nanospheres Obtained By Intramicellar Shell Crosslinking Of Polymer Micelles With Poly(ethylene Oxide) Linkers.", *Chemical Communications*, pp. 1415–1416, 1998.

34. Kim, Y., M.H. Pourgholami, D.L. Morris, and M.H. Stenzel, "Triggering The Fast Release Of Drugs From Crosslinked Micelles In An Acidic Environment.", *Journal of Materials Chemistry*, Vol. 21, pp. 12777, 2011.

35. Duong, H.T.T., C.P. Marquis, M. Whittaker, T.P. Davis, and C. Boyer, "Acid Degradable And Biocompatible Polymeric Nanoparticles For The Potential Codelivery Of Therapeutic Agents.", *Macromolecules*, Vol. 44, pp. 8008–8019, 2011.

36. Akiyoshi, K., A. Ueminami, S. Kurumada, and Y. Nomura, "Self-association Of Cholesteryl-bearing Poly(L-lysine) In Water And Control Of Its Secondary Structure By Host-guest Interaction With Cyclodextrin.", *Macromolecules*, Vol. 33, pp. 6752–6756, 2000.

37. Lee, K.Y., W.H. Jo, I.C. Kwon, Y. Kim, and S.Y. Jeong, "Structural Determination And Interior Polarity Of Self-Aggregates Prepared From Deoxycholic Acid-Modified Chitosan In Water.", *Macromolecules*, Vol. 31, pp. 378–383, 1998.

38. Akiyoshi, K., S. Deguchi, N. Moriguchi, S. Yamaguchi, and J. Sunamoto, "Self-aggregates Of Hydrophobized Polysaccharides In Water. Formation And

Characteristics Of Nanoparticles.", *Macromolecules*, Vol. 26, pp. 3062–3068, 1993.

39. López-León, T., E.L.S. Carvalho, B. Seijo, J.L. Ortega-Vinuesa, and D. Bastos-González, "Physicochemical Characterization Of Chitosan Nanoparticles: Electrokinetic And Stability Behavior.", *Journal of Colloid and Interface Science*, Vol. 283, pp. 344–351, 2005.

40. Kolb, H.C., M.G. Finn, and K.B. Sharpless, "Click Chemistry: Diverse Chemical Function From A Few Good Reactions.", *Angewandte Chemie - International Edition*, Vol. 40, pp. 2004–2021, 2001.

41. Chen, W., K. Achazi, B. Schade, and R. Haag, "Charge-conversional And Reduction-sensitive Poly(vinyl Alcohol) Nanogels For Enhanced Cell Uptake And Efficient Intracellular Doxorubicin Release.", *Journal of Controlled Release*, Vol. 205, pp. 15–24, 2015.

42. Mather, B.D., K. Viswanathan, K.M. Miller, and T.E. Long, "Michael Addition Reactions In Macromolecular Design For Emerging Technologies.", *Progress in Polymer Science (Oxford)*, Vol. 31, pp. 487–531, 2006.

43. Singh, S., J. Blöhbaum, M. Möller, and A. Pich, "Biohybrid Nanogels By Crosslinking Of Ovalbumin With Reactive Star-PEGs In W/O Emulsions.", *Journal of Polymer Science, Part A: Polymer Chemistry*, Vol. 50, pp. 4288–4299, 2012.

44. Hashimoto, Y., S. atsu Mukai, S. ichi Sawada, Y. Sasaki, and K. Akiyoshi, "Nanogel Tectonic Porous Gel Loading Biologics, Nanocarriers, And Cells For Advanced Scaffold.", *Biomaterials*, Vol. 37, pp. 107–115, 2015.

45. Singleton, D.A., D.T. Nowlan, N. Jahed, and K. Matyjaszewski, "Isotope Effects And The Mechanism Of Atom Transfer Radical Polymerization.", *Macromolecules*, Vol. 36, pp. 8609–8616, 2003.

46. Tang, W., Y. Kwak, W. Braunecker, N. V. Tsarevsky, M.L. Coote, and K. Matyjaszewski, "Understanding Atom Transfer Radical Polymerization: Effect Of Ligand And Initiator Structures On The Equilibrium Constants.", *Journal of the American Chemical Society*, Vol. 130, pp. 10702–10713, 2008.

47. Kim, Y., S.O. Ho, N.R. Gassman, Y. Korlann, E. V. Landorf, F.R. Collart, and S. Weiss,

"Efficient Site-specific Labeling Of Proteins Via Cysteines.", *Bioconjugate Chemistry*, Vol. 19, pp. 786–791, 2008.

48. Hall, D.J., H.M. Van Den Berghe, and A.P. Dove, "Synthesis And Post-polymerization Modification Of Maleimide-containing Polymers By 'Thiol-ene' Click And Diels-Alder Chemistries.", *Polymer International*, Vol. 60, pp. 1149–1157, 2011.

49. Subramani, C., N. Cengiz, K. Saha, T.N. Gevrek, X. Yu, Y. Jeong, A. Bajaj, A. Sanyal, and V.M. Rotello, "Direct Fabrication Of Functional And Biofunctional Nanostructures Through Reactive Imprinting.", *Advanced Materials*, Vol. 23, pp. 3165–3169, 2011.

50. Koehler, K.C., K.S. Anseth, and C.N. Bowman, "Diels-alder Mediated Controlled Release From A Poly(ethylene Glycol) Based Hydrogel.", *Biomacromolecules*, Vol. 14, pp. 538–547, 2013.

51. Park, E.J., T.N. Gevrek, R. Sanyal, and A. Sanyal, "Indispensable Platforms For Bioimmobilization: Maleimide-based Thiol Reactive Hydrogels.", *Bioconjugate Chemistry*, Vol. 25, pp. 2004–2011, 2014.

52. Arslan, M. Sanyal, R., P. Woisel, and A. Sanyal, "Bioinspired Anchorable Thiol-Reactive Polymers: Synthesis And Applications Toward Surface Functionalization Of Magnetic Nanoparticles.", *Macromolecules*, pp. 5125–51334, 2014.

53. Mantovani, G., F. Lecolley, L. Tao, D.M. Haddleton, J. Clerx, J.J.L.M. Cornelissen, and K. Velonia, "Design And Synthesis Of N-maleimido-functionalized Hydrophilic Polymers Via Copper-mediated Living Radical Polymerization: A Suitable Alternative To Pegylation Chemistry.", *Journal of the American Chemical Society*, Vol. 127, pp. 2966–2973, 2005.

54. Dispinar, T., R. Sanyal, and A. Sanyal, "A Diels-Alder/retro Diels-Alder Strategy To Synthesize Polymers Bearing Maleimide Side Chains.", *Journal of Polymer Science, Part A: Polymer Chemistry*, Vol. 45, pp. 4545–4551, 2007.

55. Hanay, S.B., "*Thiol Reactive Hydrogels From Single Precursor*", Istanbul, 2011.

56. Gok, O., I. Kosif, T. Dispinar, T.N. Gevrek, R. Sanyal, and A. Sanyal, "Design And Synthesis Of Water-Soluble Multifunctionalizable Thiol-Reactive Polymeric Supports For Cellular Targeting.", *Bioconjugate chemistry*, Vol. 26, pp. 1550–60, 2015.

57. Amat-Guerri, F., M. Liras, M.L. Carrascoso, and R. Sastre, “Methacrylate-tethered Analogs Of The Laser Dye PM567--synthesis, Copolymerization With Methyl Methacrylate And Photostability Of The Copolymers.”, *Photochemistry and photobiology*, Vol. 77, pp. 577–584, 2003.

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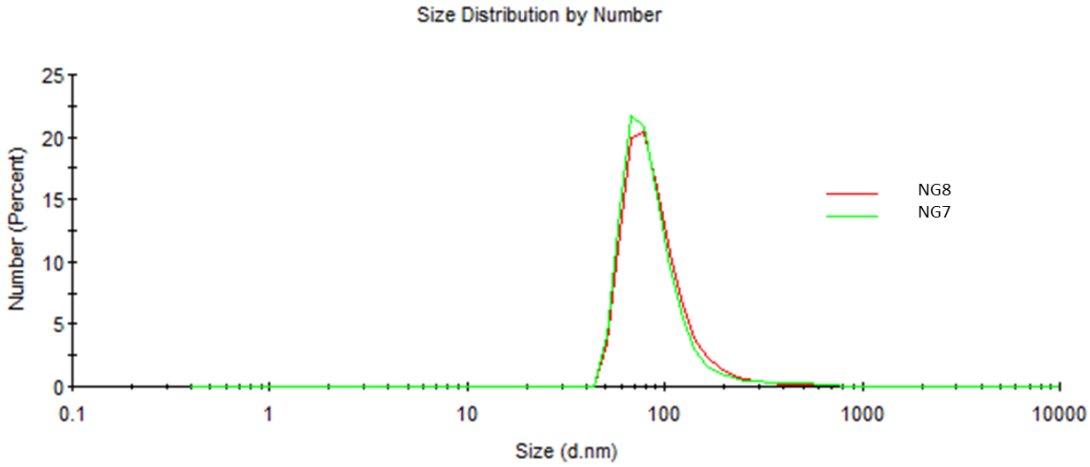

Figure A-5.1. DLS results of NG8 and NG7

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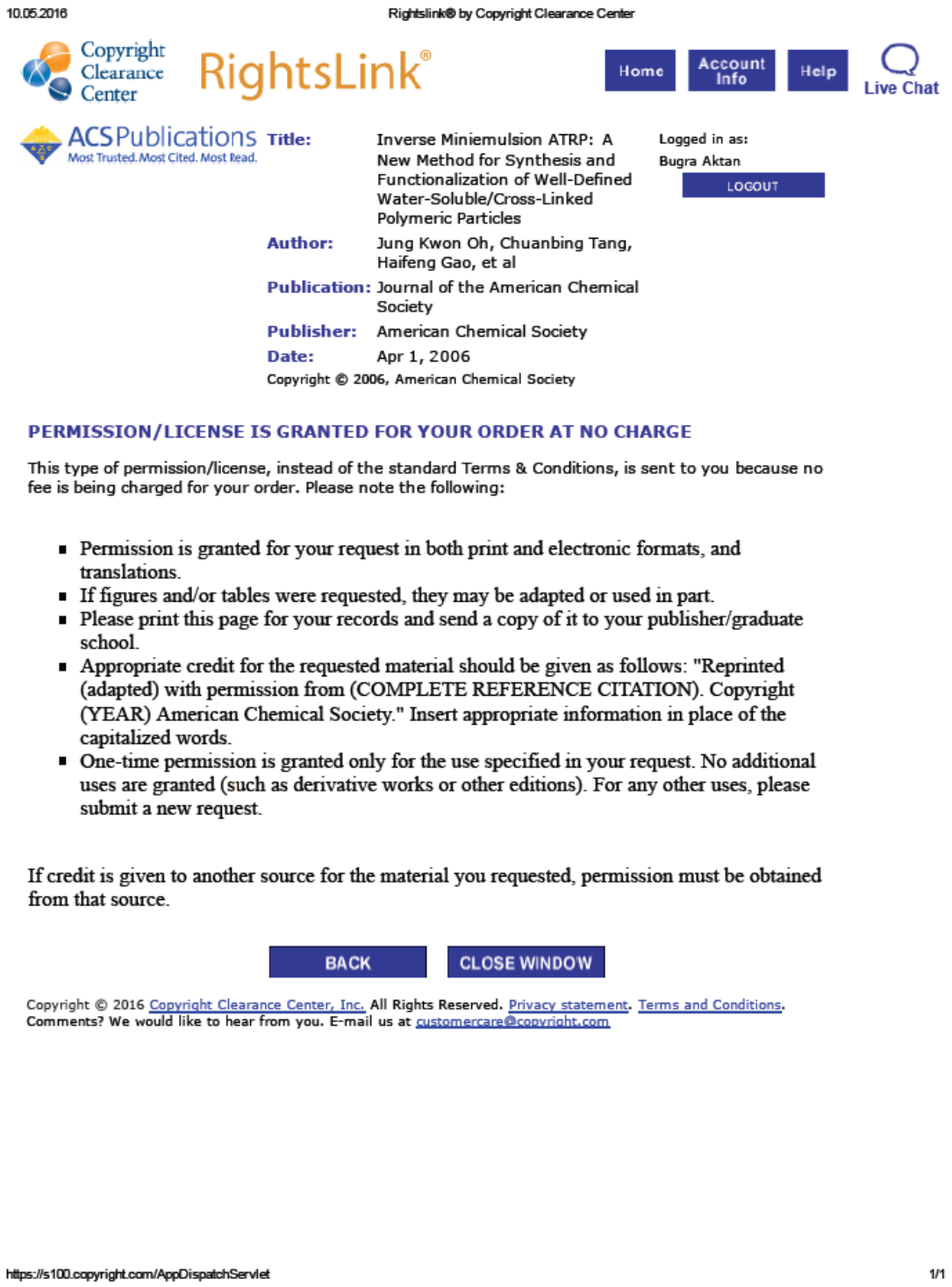

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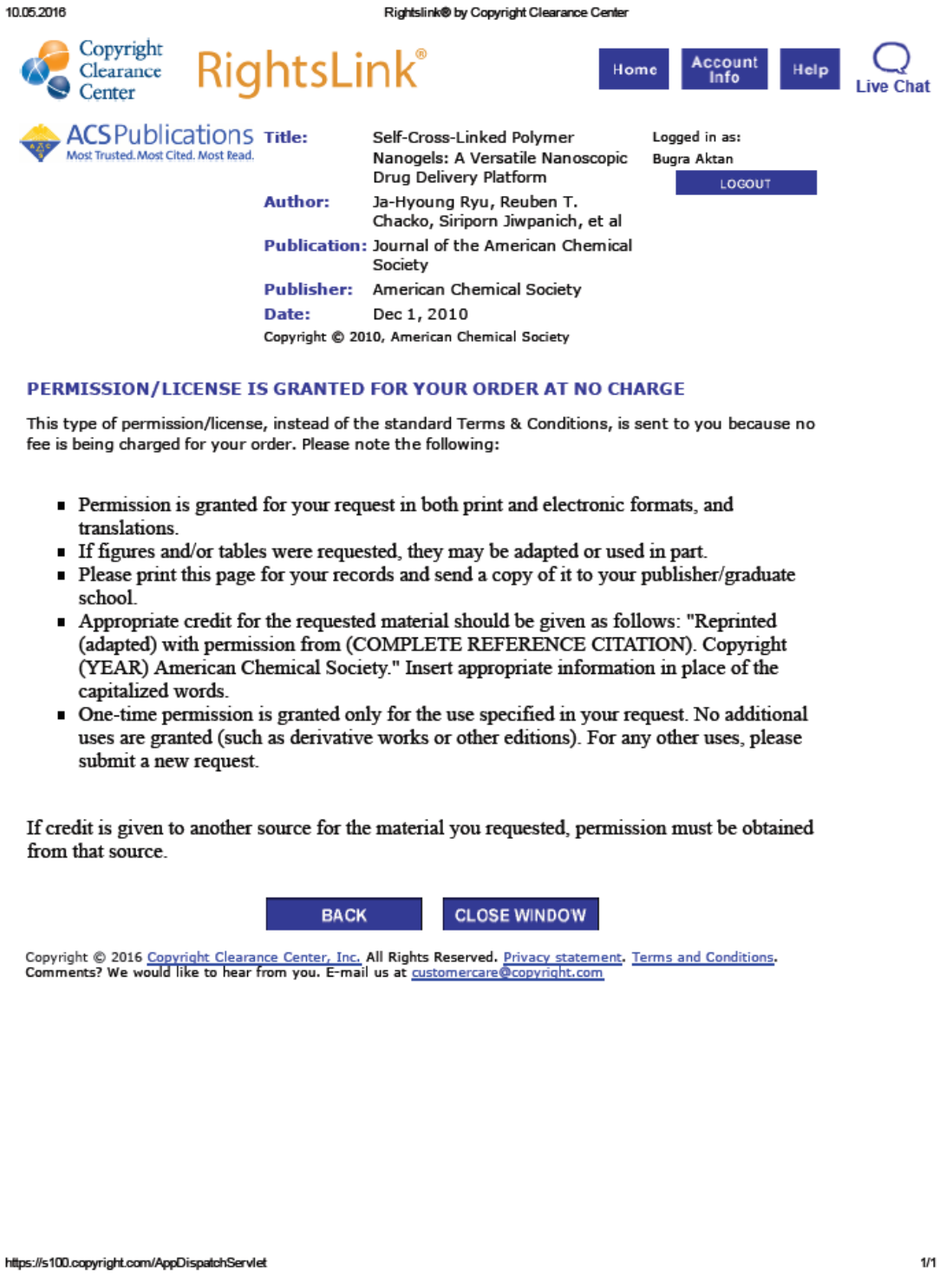

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Author: Ja-Hyoung Ryu, Reuben T. Chacko, Siriporn Jiwpanich, et al

Publication: Journal of the American Chemical Society

Publisher: American Chemical Society

Date: Dec 1, 2010

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