SYNTHESIS AND APPLICATION OF THIOL-REACTIVE ZWITTERIONIC COPOLYMERS

by

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ABSTRACT

SYNTHESIS AND APPLICATION OF THIOL-REACTIVE ZWITTERIONIC COPOLYMERS

In recent years, zwitterionic polymeric materials have gained attention thanks to their ultra-hydrophilicity due to the equimolar number of evenly distributed anionic and cationic moieties that they contain along their polymer chains while still preserving their overall charge neutrality. Moreover, zwitterionic polymers are typically regarded as an alternative to the popular poly(ethylene glycol) (PEG) polymers for anti-biofouling applications to prevent nonspecific protein adsorption and to reduce bacterial or mammalian cell adhesion.

Due to its potential uses in interdisciplinary fields, including biomaterials, material science, and targeted delivery, the synthesis of zwitterionic polymers with reactive functional groups is a popular area of research. Polymers with reactive maleimide units as functional groups at the side chains can be obtained by a copolymerization technique using zwitterionic monomers and maleimide-containing monomers.

In the thesis, the functionalization of zwitterionic structures was done by copolymerization with an acrylate-based 'latent-reactive' monomer in which maleimide is masked with furan by the Diels-Alder reaction to protect the double bond of the maleimide moiety. By using the advantage of the thermo-responsive behavior of the cycloadduct, the thiol-reactive maleimide unit was activated by the retro DA reaction. To show the effectiveness of the maleimide unit, the thiol-containing hydrophobic dye was conjugated to the linear polymeric structure by the 'click' reaction. Since the hydrophobic dye can become soluble in water when attached to the designed zwitterionic polymer, the methodology serves as a model of drug conjugation.

ÖZET

TİOL-REAKTİF ZWİTTERİYONİK KOPOLİMERLERİN SENTEZİ VE UYGULAMASI

Son yıllarda, zwitteriyonik polimerik malzemeler, genel yük nötrlüklerini korurken polimer zincirleri boyunca içerdikleri eşit mol sayısındaki eşit olarak dağılmış anyonik ve katyonik kısımlar sayesinde kazandıkları ultra hidrofiliklik özellikleri ile dikkat çekmiştir. Ayrıca, zwitteriyonik polimerler tipik olarak, spesifik olmayan protein adsorpsiyonunu önlemek ve bakteri veya memeli hücre yapışmasını azaltmak için biyokirlenmeyi önleme uygulamalarında kullanılan popüler poli(etilen glikol) (PEG) polimerlerine alternatif olarak kabul edilmektedir.

Biyomalzemeler, malzeme bilimi ve hedeflenen teslimat dahil olmak üzere disiplinler arası alanlardaki potansiyel kullanımları nedeniyle, reaktif fonksiyonel gruplarla zwitteriyonik polimerlerin sentezi popüler bir araştırma alanıdır. Yan zincirlerde fonksiyonel gruplar olarak reaktif maleimid birimlerine sahip polimerler, zwitteriyonik monomerler ve maleimid içeren monomerler kullanılarak bir kopolimerizasyon tekniği ile elde edilebilir.

Tezde, zwitteriyonik yapıların işlevselleştirilmesi, maleimidin çift bağını korumak için Diels-Alder reaksiyonu ile maleimidin furan ile karıştırıldığı yeni bir akrilat bazlı 'gizli reaktif' monomer ile kopolimerizasyon yoluyla yapıldı. Siklo katkı ürününün ısıya duyarlı davranışının avantajını kullanarak, tiyol reaktif maleimid birimi, retro Diels-Alder (DA) reaksiyonu ile aktive edildi. Maleimid biriminin etkinliğini göstermek için, tiyol içeren hidrofobik boya, lineer polimerik yapıya “klik” reaksiyonu ile konjuge edildi. Hidrofobik boya, tasarlanan zwitteriyonik polimere eklendiğinde suda çözünür hale gelebildiğinden, metodoloji bir ilaç konjugasyonu modeline de hizmet eder.

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LIST OF ACRONYMS/ABBREVIATIONS

ACVA	4,4'-Azobis(4-cyanovaleric acid)
ALCAM	Activated Leukocyte Cell Adhesion Molecule
ATRP	Atom Transfer Radical Polymerization
$CDCl_3$	Chloroform
CH_2Cl_2	Dichloromethane
CTA	4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid
D_2O	Deuterated Water
DA	Diels-Alder
Da	Dalton
DMAP	4-(Dimethylamino)pyridine
FTIR	Fourier Transform Infrared
kDa	Kilo Dalton
MeOH	Methanol
MHz	Mega Hertz
M_n	Number Average Molecular Weight
MWCO	Molecular Weight cut-off
NMP	Nitroxide-Mediated Polymerization
NMR	Nuclear Magnetic Resonance
PCB	Polycarboxybetaines
PDI	Polydispersity Index
PEG	Poly(ethylene glycol)
PEGMEMA	Poly(ethylene glycol) monomethyl ether methacrylate
PNA	Peptide Nucleic Acid
RAFT	Reversible Addition-Fragmentation Chain Transfer Polymerization
RDRP	Reversible-Deactivation Radical Polymerization
ROMP	Ring-Opening Metathesis Polymerization
rt	Room Temperature
SBMA	N-(3-Sulfopropyl)-N-methacroyl-oxyethyl-N,N-dimethylammonium betaine

TEA	Triethylamine
TFE	2,2,2-Trifluoroethanol
THF	Tetrahydrofuran
UV	Ultraviolet
Vis	Visible

1. INTRODUCTION

1.1. Zwitterionic Polymers

The polymers that contain oppositely charged ions, in contrast to polyelectrolytes in which the monomers are either polycations or polyanions, in their structures are called polyzwitterions or zwitterionic polymers [1]. Various options exist for the negative and positive charges to be spaced along the chain. The ones where different charges are found on different monomer units are called polyampholytes, for which typical examples are proteins and peptides. In contrast, polybetaines are those in which the monomers are zwitterionic (Figure 1.1.a, b). Sulfobetaines, carboxybetaines, and phosphobetaines are examples of such monomers (Figure 1.1.c). For obtaining the zwitterionic structures, there are several ways to incorporate ion pairs into polymers (Figure 1.2). Because of the complexity of their synthesis, polymers with charged backbones rather than side chains are comparatively rare [2].

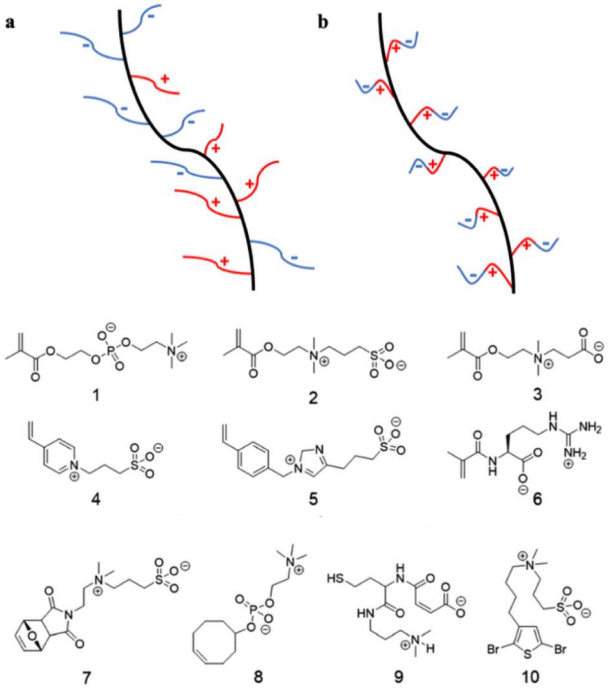

Figure 1.1. Schematic representation of (a) a polyampholyte, (b) a polybetaine, and (c) chemical structures of commonly used zwitterionic monomers [2].

Depending on the stoichiometric ratio of anionic and cationic structures, the overall charge on the zwitterionic polymer might be zero or vary from zero. By changing the ambient conditions, polyelectrolytic behavior can be imparted to zwitterionic polymers allowing for the development of stimuli-responsive materials. Both the stoichiometry and the spacing of the charges along the polymer have a significant impact on the behavior of the polymer and thus on the applications of the material. The main factor that affects the behavior of the polyampholytes or polybetaines is their pH sensitivity, which can be better understood by determining the isoelectronic point of the polymers. The overall net charge of polymers is zero when they reach their isoelectronic point, at which their solubility decreases due to their global conformation. As either the cationic or the anionic charges become dominant due to shifts in pH, coil conformation is reached owing to the repelling of like charges, enhancing the polymer's solubility (Figure 1.3). In contrast to alternating polyampholytes, those with a long spacing of opposite charges, as in diblock polyampholytes, show a coil-to-globule transition causing a decrease in solubility [2].

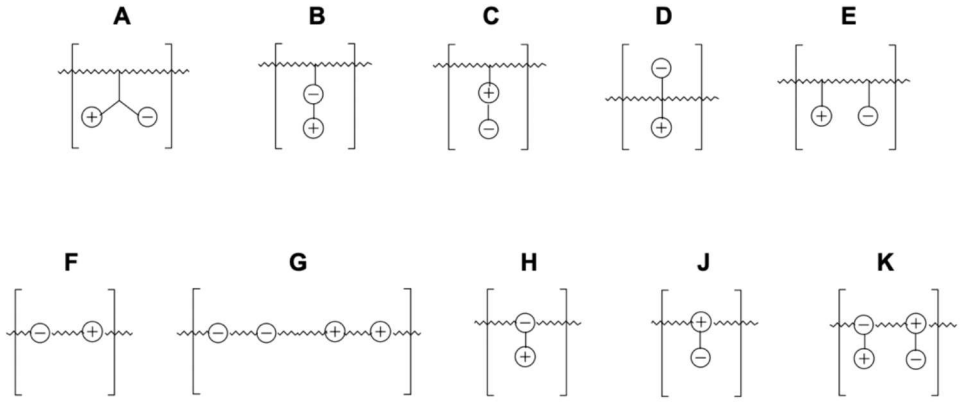

Figure 1.2. The potential arrangement of ionic groups throughout polyzwitterions [3].

In marine engineering and the biomedical industry, biofouling is a significant problem [4]. Biofouling is the specific detection of nontargeted molecules such as proteins, microorganisms, or biofilms on the specifically designed materials used in implants, biosensors, or drug delivery systems. To alleviate the fouling issue, designing materials from hydrophilic polymers, in which interaction between the surface of the material and the foiling substance is reduced, is an effective method. Poly(vinyl alcohol), poly(N-vinylpyrrolidone), poly(2-oxazo-line), and poly(ethylene glycol) (PEG) are examples of

such hydrophilic polymers, among which PEG polymers are the ones that are most commonly used thanks to their non-toxic, anti-biofouling and highly hydrophilic properties. However, due to the degradation and oxidation of the chains by the metal catalysts, the stability of PEG polymers is considered poor, limiting its long-term *in vivo* usage [5]. Moreover, PEG-based brush structures lose their antifouling property at above 35 °C [6].

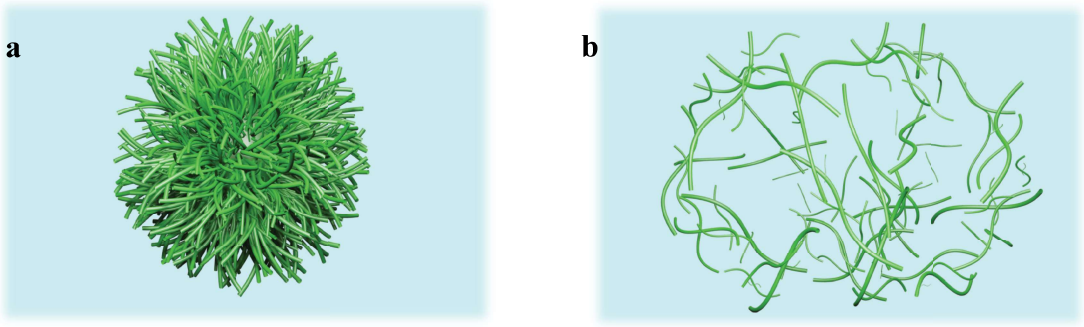

Figure 1.3. Schematic representation of (a) zwitterionic polymer at the isoelectric point forming a global conformation and (b) zwitterionic polymer below or above the isoelectronic point forming a coil conformation.

PEG-based polymers offer many uses in drug delivery, biosensing, imaging, etc. PEGylation technique, for instance, meaning the conjugation of PEG to either proteins, peptides, nucleotides, or nanomaterials that encapsulate the drug, has been used in clinics for over 40 years. It's becoming increasingly clear that giving PEGylated drugs to patients can cause antibodies to develop that identify and bind to PEG. Anti-PEG bodies are not found only in those treated with the drugs but in those used products that contain PEG. Due to this phenomenon, the benefits are apparent only in the initial dosage, which reduces the drug's efficacy. Because the mononuclear phagocyte system detects quick clearance in the second dosage, PEGylated drug or nanomaterial treatments result in accelerated drug release in patients who have acquired anti-PEG antibodies. Zwitterionic polymers are gold-standard alternatives for PEG polymers to address those problems since zwitterionic polymers are thermally and chemically stable, not recognized by the immune system, resistant to nonspecific protein fouling in complex media, and super hydrophilic compared to PEG [7]. In addition, polysulfobetaines and polycarboxybetaines, which can be used to prepare multifunctional nanoparticles, are easy to manufacture. Environmentally friendly hydrolyzable zwitterionic esters are potential materials to prepare antibacterial coatings and

gene-delivery carriers. Consequently, it is promising to use mixed-charge species to make a wide range of antifouling materials [8].

1.2. Synthesis of Zwitterionic Polymers

Polymerization of zwitterionic monomers, as well as post-polymerization modifications, are all possible ways to make polyampholytes. Those various ways are developed due to practical issues such as difficulties in obtaining a stable zwitterionic monomer, the reactivity of the zwitterionic fraction, or solubility problems [9, 10]. The zwitterionic moiety of the monomer might be involved in a chemical reaction by being a reactant or an inhibitor during polymerization, in which case protection of the zwitterionic group is needed (Figure 1.4, path 2, 3). Moreover, finding an appropriate solvent can be challenging due to the strong intra- and intermolecular electrostatic interactions. Polar protic solvents, aqueous salt solutions, and fluorinated alcohols such as trifluoroethanol (TFE) and hexafluoroisopropanol [11] are some examples that dissolve the zwitterionic structures entirely since they have a strong ability to block the electrostatic interactions [12]. Despite their high cost and the difficulty of completely removing the solvent from the medium after an efficient polymerization, ionic liquids are great alternatives to dissolving polymers in which the hydrophilic and hydrophobic components are present, as in the example of copolymers [3, 13].

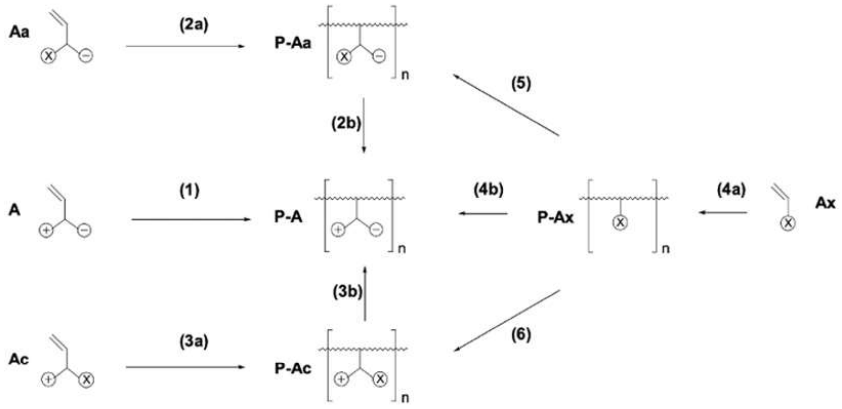

Figure 1.4. Possible polyzwitterion synthesis routes are shown for architectural type A in Figure 1.2 [3].

1.2.1 Synthesis of Zwitterionic Polymers by Chain-Growth Polymerization

Free radical polymerization, a type of chain-growth polymerization, is commonly preferred in synthesizing zwitterionic polymer because the radical polymerization can occur in the presence of electrophilic and nucleophilic moieties concurrently of zwitterionic monomers [14, 15]. The most common commercially available zwitterionic monomers used in such polymerization are methacrylic sulfobetaine and phosphatidylcholine monomers (Figure 1.5) [3].

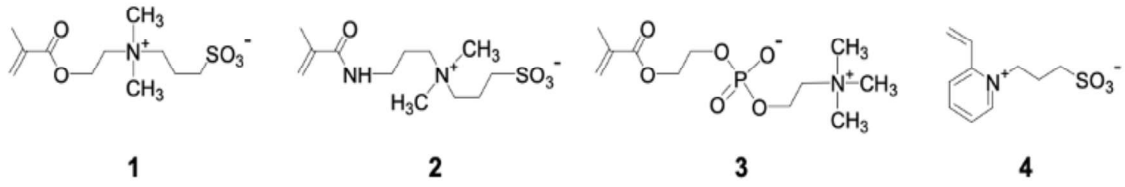

Figure 1.5. Commercially available polymers that can be polymerized directly [3].

As an example of a chain-growth polymerization technique, reversible-deactivation radical polymerization (RDRP) has sparked a lot of creative and original work over the last 15 years. The predetermined molecular weight and the low polydispersity index (PDI) are two advantages of the RDRP technique [16]. Moreover, zwitterionic polymers can be functionalized from the reactive end-groups [17, 18], or applications can be varied by using complex polymeric structures such as block copolymers [19, 20], graft copolymers [21, 22], hyperbranched polymers [23], or star polymers [24, 25]. Thus, the development of polyzwitterions with new polymer architectures using RDRP techniques has increased dramatically, whether through nitroxide-mediated polymerization (NMP) [26, 27], atom transfer radical polymerization (ATRP) [28, 29], reversible addition-fragmentation chain transfer polymerization (RAFT) [30, 31], or ring-opening metathesis polymerization (ROMP) [32, 33]. Importantly, RDRP methods are also used to conjugate specific biomolecules by using reactive groups [34, 35], which are often crucial for designing biomaterials or sensors. For industrial applications, grafting-from [36, 37], grafting-to [38, 39], and grafting-through [38, 40] procedures are in use.

Yuan J. et al., for instance, used the RAFT technique to graft sulfobetaine-based zwitterionic polymer onto the surface. First, the chain transfer agent was attached to the surface by esterification. Surface initiation was used to grow zwitterionic polymers on the surface to enhance anti-biofouling and hemocompatibility (Figure 1.6) [31].

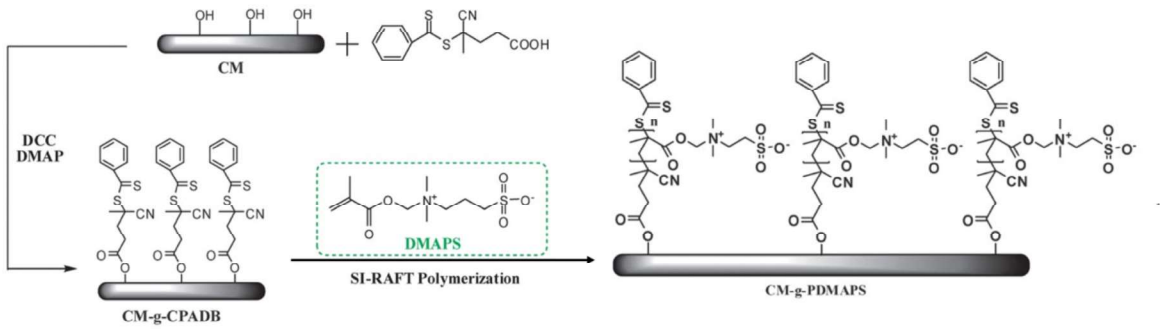

Figure 1.6. Attachment of zwitterionic structure onto the cellulose surface by surface initiated-RAFT technique [31].

To synthesize fluorescent biocompatible copolymers, Madsen, J. et al designed a dye-conjugated ATRP initiator to polymerize a phosphobetaine zwitterionic monomer. The covalent attachment of the dye to the zwitterionic polymer enables their monitoring in cells. The designed system was functionalized by adding a pH-responsive monomer resulting in a block copolymer (Figure 1.7) [28].

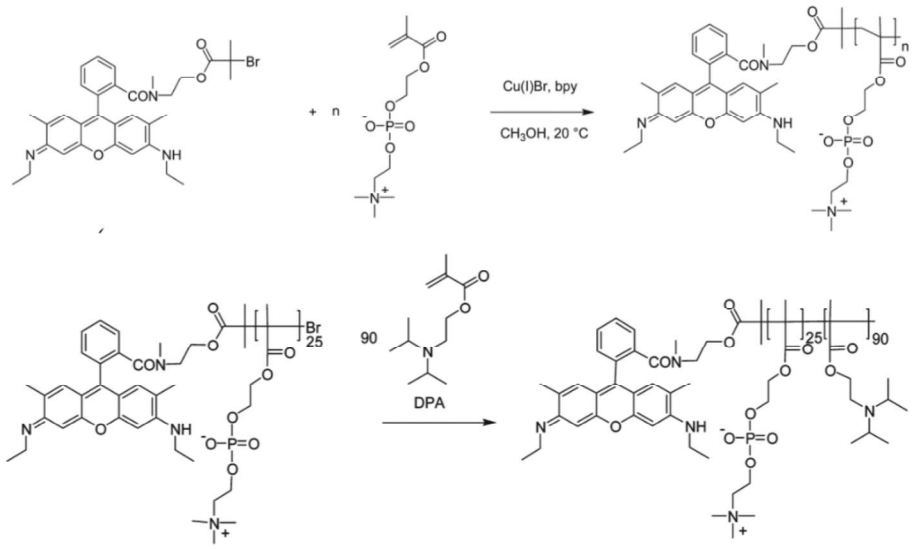

Figure 1.7. Synthesis of pH-responsive fluorescent zwitterionic block copolymer by ATRP [28].

1.2.2. Synthesis of Zwitterionic Polymers by Step-Growth Polymerization

Step growth methods are rarely used to synthesize polyzwitterions [3], while numerous ways have been described in Figure 1.8 as the synthesis using polyurethanes [41], polycarbonates [42], polysiloxanes [43], and conjugated backbones [44]. The utilization of zwitterionic monomers in such polymerizations is complicated due to hygroscopy, a preference for nucleophilic and protic solvents for their solubility, and high melting temperatures because of their ionic nature [3].

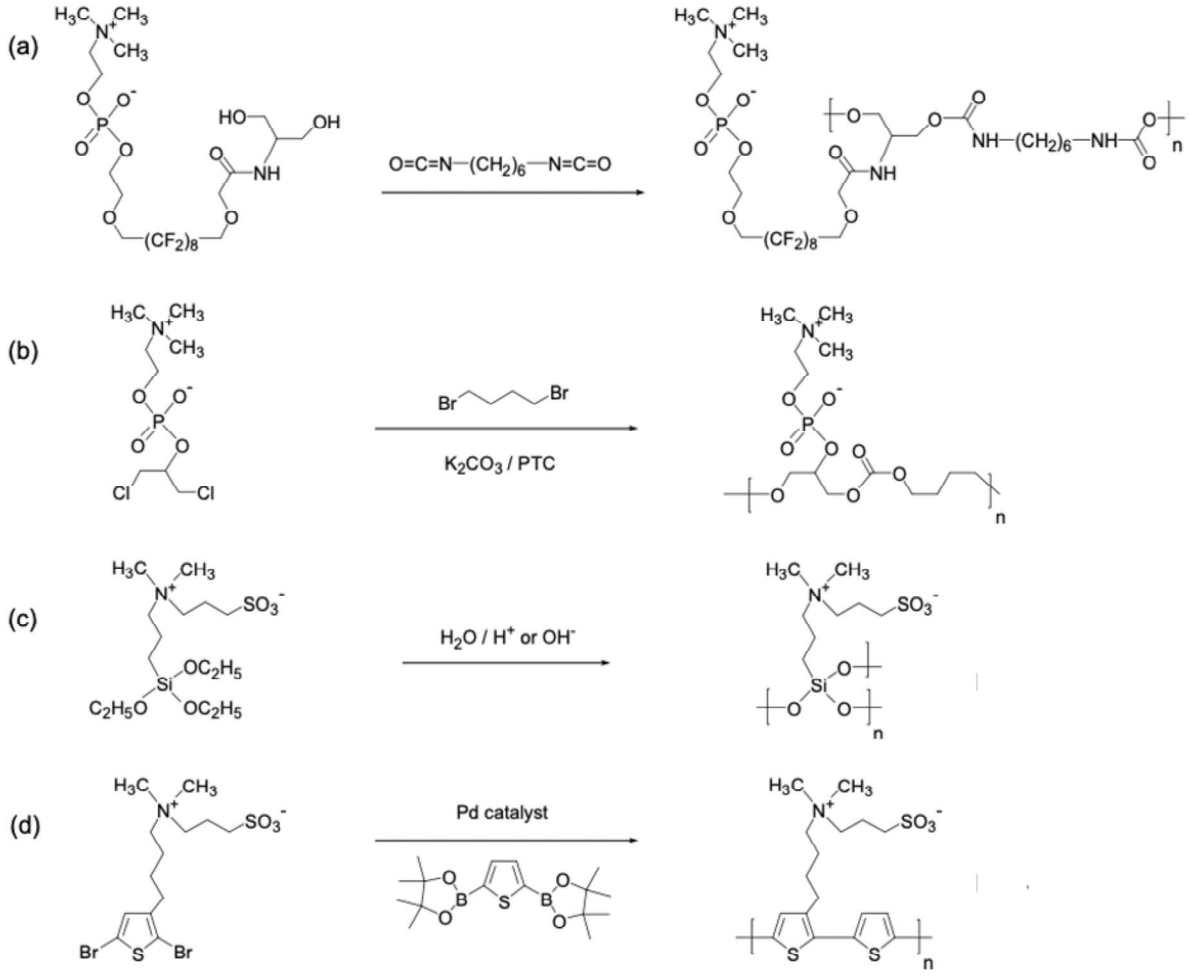

Figure 1.8. Examples of step growth polymerization-produced polyzwitterions: (a) polyurethanes, (b) polycarbonates, (c) polysiloxanes, and (d) conjugated backbones [3].

Other documented step-growth polymerizations toward polyzwitterions (Figure 1.9) appear not as a standard method but are chemically applicable because the ionic nature of

the compounds does not affect the method, such as polymerization of polythioether via thiol-ene addition [45], polydisulfide via oxidative coupling [46], and polyester via [2 + 2] photo-cycloaddition [47].

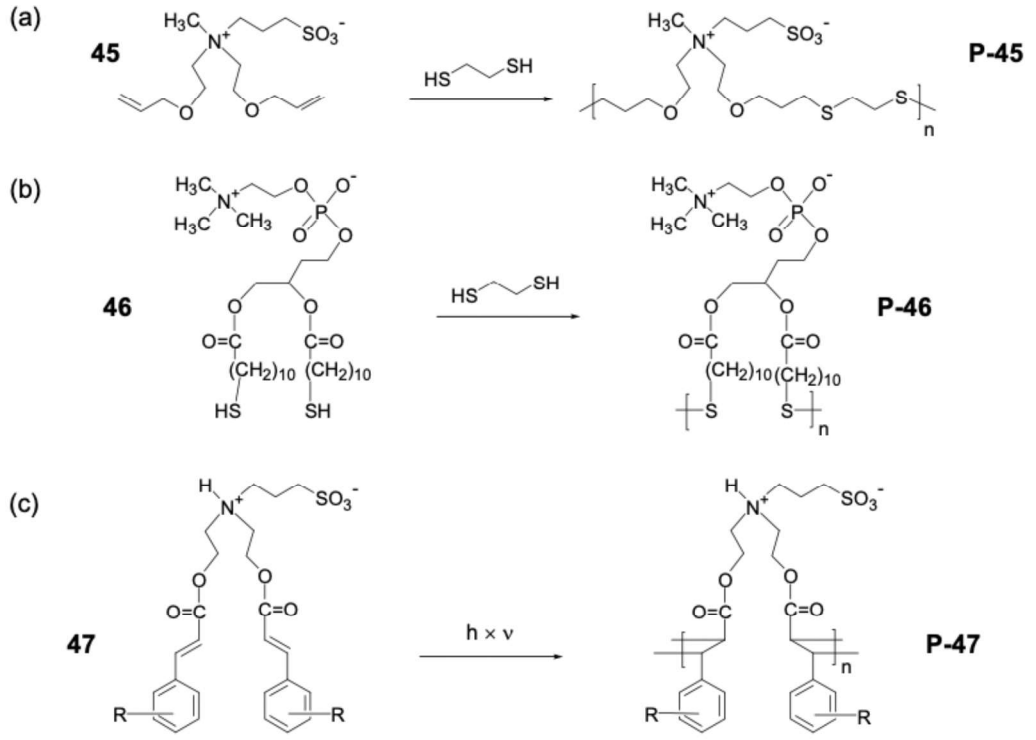

Figure 1.9. Examples for polyzwitterions produced via step-growth polymerization: (a) Polythioether via thiol-ene addition, (b) Polydisulfide via oxidative coupling, and (c) Polyester via [2 + 2] photo-cycloaddition [3].

1.2.3. Synthesis of Zwitterionic Polymers by Post-Growth Polymerization

As discussed previously, post-polymerization modification methods are encouraging in some instances due to the sensitivity, incompatibility between the ionic moiety and the polymerization technique, or solubility and characterization problems. Alkylation of polymer-bound containing a cationic structure, usually a tertiary amine, is the most used method to introduce the anionic group to synthesize a polyzwitterion. Carboxylic acids that carry halogens as leaving groups can be preferred for the alkylation. (Figure 1.4, path 3b). Bohrish J. et al. obtained polycarbobetaines by bromoesterification followed by hydrolysis

of the ethyl ester [48], in which the anionic site is protected in the first step and then released in the second step.

To be able to prepare polymeric ammonium bisphosphonate N. Hu et al. protected the anionic group as an ester, and then deprotection was done by using methanol [49]. There is always a possibility of incomplete conversion in post modifications, which disturbs the neutral environment of zwitterions. The unreacted sites can be coupled with zwitterionic reagents in which conversion will not be 100% of the time. That is why the remaining reactive sites shouldn't be the groups as carboxylic acids or amines because they are ionizable, and neutrality couldn't be achieved.

Cycloaddition of azides and alkynes is another helpful way to get zwitterions, even if there are still unreacted sites after the post-modification and coupling with the zwitterionic reagents since they are harmless residuals. Yu, X. et al. synthesized a hyperbranched polyglycerol by reacting zwitterionic alkyne with the polymeric azide (Figure 1.10) [50].

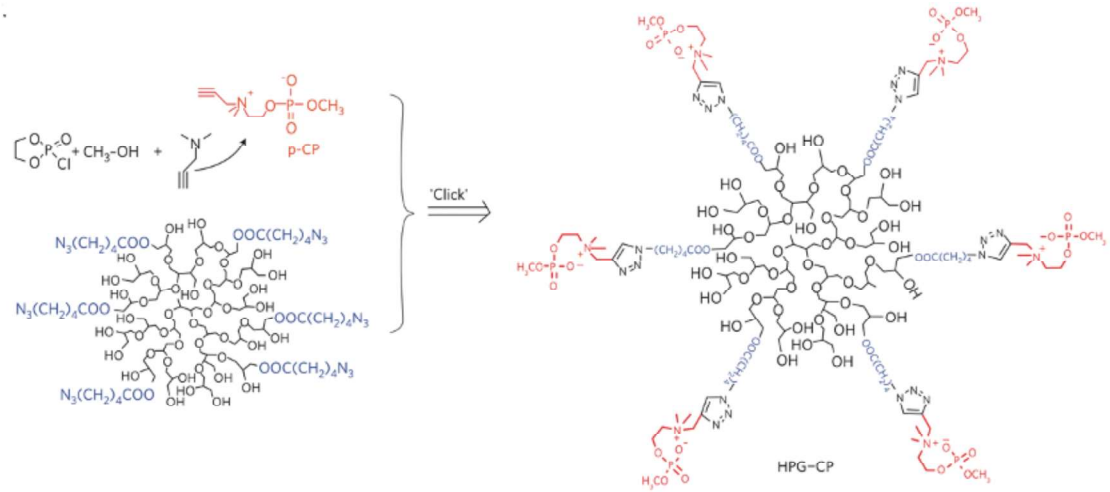

Figure 1.10. The 'click' synthesis of polyvalent choline phosphate based on hyperbranched polyglycerol and the synthesis of prop-2-ynyle choline phosphate [50].

The examples presented show immense potential for polyzwitterion synthesis by precursor polymer modification. With the different disadvantages kept in mind, it must be

determined whether this method is acceptable or advantageous based on the specific scenario and requirements.

1.3. Applications of Zwitterionic Polymers

The use of zwitterionic polymers as alternatives to PEG polymers in biomedical applications is favorable, thanks to their promising anti-biofouling capacity. Because of the inherent absence of functional groups, their biosensing use has been severely limited. One can copolymerize the zwitterions with a non-zwitterionic monomer having a functional group to overcome this issue. Wen et al. copolymerized the glycidyl methacrylate (GMA), in which epoxide is the functional group, with the sulfobetaine methacrylate (SBMA). The resulting polymer, poly(GMA-co-SBMA), is then grafted onto the nanocubes on the metal surface modified with free-SH groups, which can react with the epoxide group on the polymer. This system is designed to trap malachite green, which binds to water molecules found around the $^{-}SO_3$ and C_4N^+ sites on the copolymer grafted surface from platelet-rich plasma (Figure 1.11) [51].

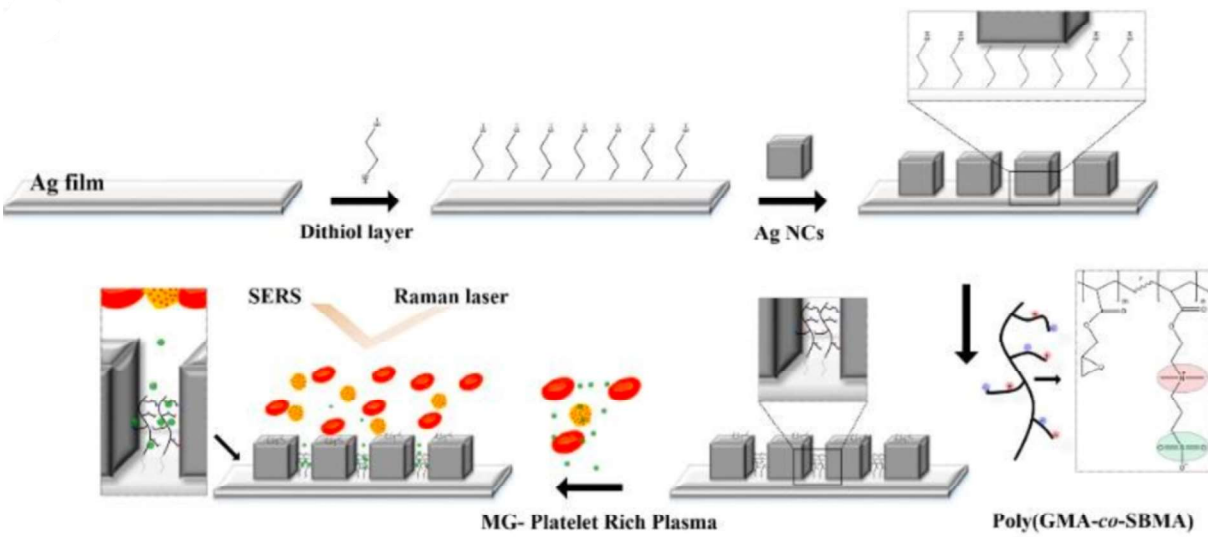

Figure 1.11. Illustrations of surface modification of poly(glycidyl methacrylate-co-sulfobetaine methacrylate [51].

To design a sensor, Zuilhof et al. constructed a sulfobetaine-based zwitterionic monomer with an azide moiety so that both the functionality and the anti-biofouling property can be found in a single monomer structure. A diamine is first monoprotected, then the free

amine is converted into the azide to synthesize a novel monomer. The alkylation of the tertiary amine brings the zwitterionic character to the structure. In the final step, deprotection is followed by introducing the polymerizable methacrylamide unit. The copolymerization of the resulting monomer with the standard SBMA results in a polymer brush coating with an anti-biofouling surface and a high number of clickable groups coupled with an alkyne to be used in any specific recognition application (Figure 1.12) [52].

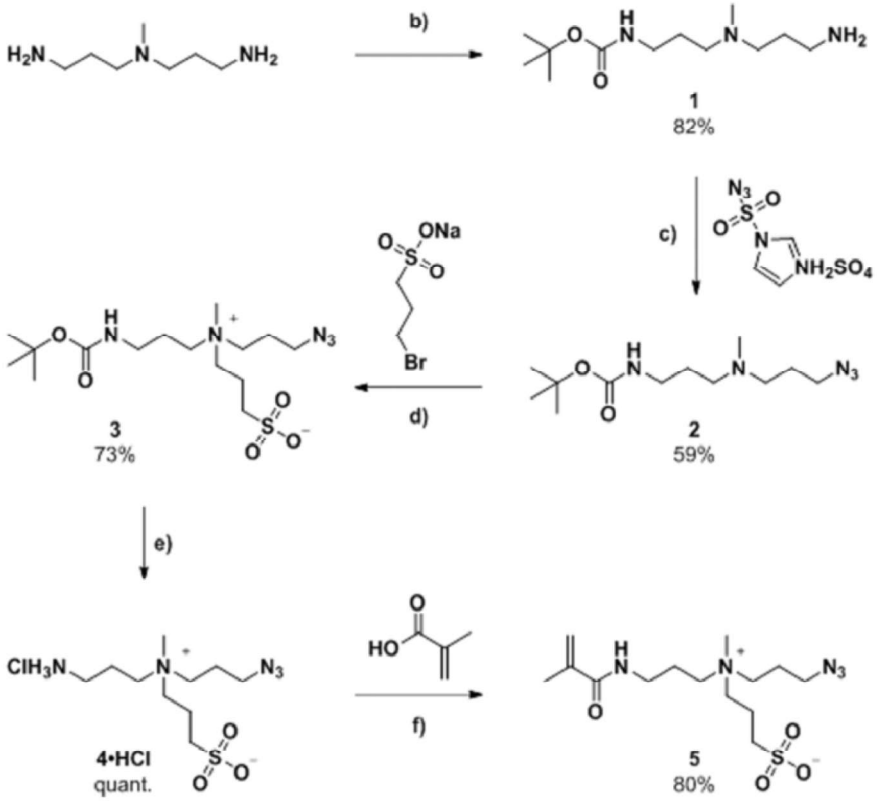

Figure 1.12. Synthesis of sulfobetaine-based zwitterionic monomer in which b represents Boc_2O, and e represents HCl [52].

For the modification of sensors, polycarboxybetaines (PCB) are the most commonly used zwitterions, especially in cancer detection, because of the abundant -COOH, which later can be functionalized. Activated leukocyte cell adhesion molecule (ALCAM) levels in various carcinomas are known to be greater than the usual level of ALCAM in blood serum, which means the detection of ALCAM on the surface is crucial to detecting cancer [53]. To achieve this goal, the surface should be highly anti-biofouling. Jiang et al. grafted the (3,4-Dihydroxy-L-phenylalanine)2-(poly(carboxybetaine methacrylate)2) (DOPA2-pCBMA2) polymer onto the silica surface. The reactive NHS esters are produced from the terminal

carboxylic acids and bind to the primary amines on IgG antibodies. The number of active ALCAM binding sites is considered linearly proportional to the level of immobilized anti-ALCAM IgG. ALCAM is detected in diluted serum with a detection limit of 10 ng/mL(Figure 1.13) [54].

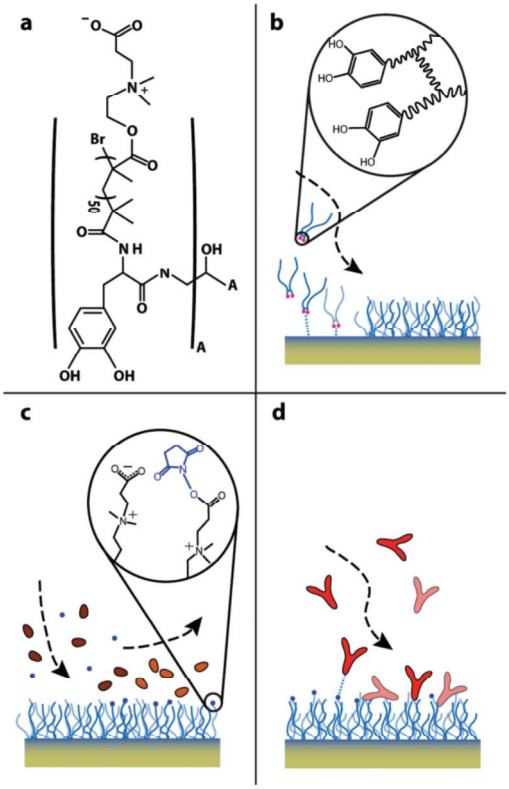

Figure 1.13. Adsorption of pCBMA2 by DOPA2 and functionalization of antibodies. Structures, two of which are joined by an R- COH - R bridge, are denoted by the letter “A” [54].

For the DNA isolation, Poly-[(propargyl methacrylate)-ran-(2-methacryloyloxyethyl phosphorylcholine)] (PPgMAMPC) was synthesized by reversible addition-fragmentation polymerization, and then dithioester groups at the chain ends are converted to thiol by the aminolysis reaction with hydrazine monohydrate so that the copolymer can be immobilized to the gold-coated surface by the “grafting-to” method via self-assembly. The alkyne moiety in propargyl methacrylate makes the polymer react to azide-containing compounds. Wiarachai, O. et al. applied this feature to biosensing applications, attaching azide-containing species such as biotin and peptide nucleic acid (PNA) to detect streptavidin

(SA) and DNA, respectively [55]. Peptide nucleic acids (PNAs) are artificial DNA replicas in which the nucleobases have been linked to a pseudo-peptide polymer in place of the deoxyribose phosphate backbone [56], which binds to DNA by hydrogen bonding. Due to the neutral environment of PNA, electrostatic repulsion between negatively charged phosphate groups in the DNA backbone are absent, which results in stronger interactions of PNA-DNA hybrids compared to DNA-DNA hybrids (Figure 1.14) [55].

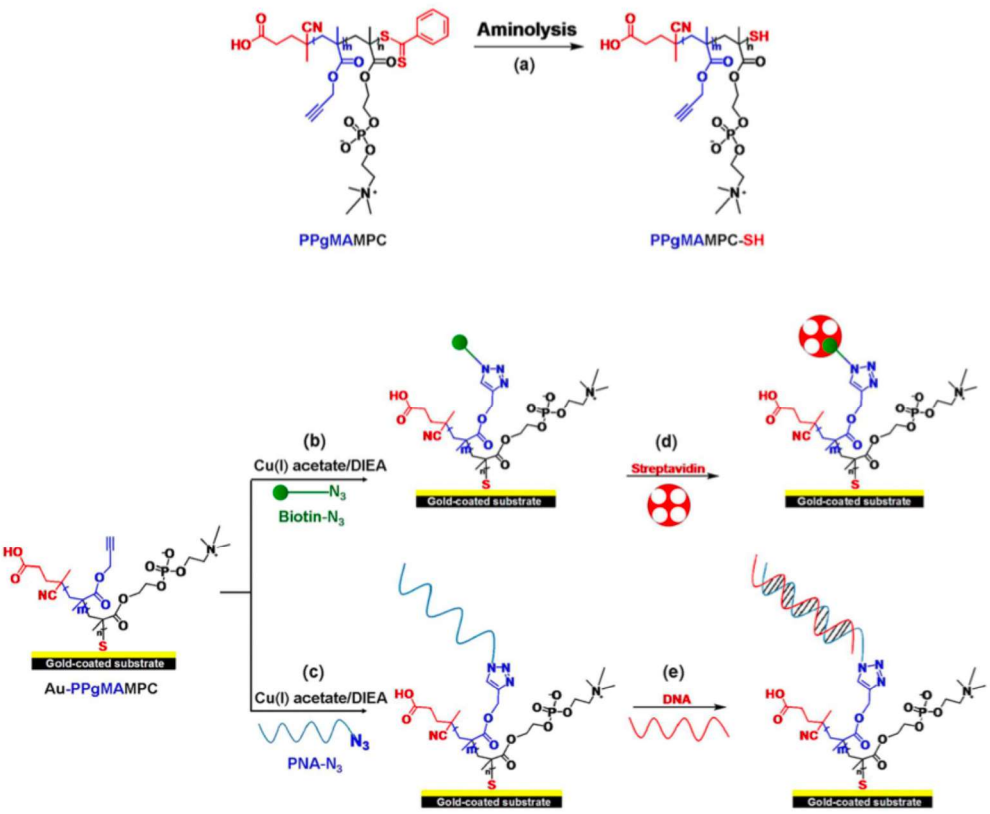

Figure 1.14. Schematic diagram of (a) preparation of thiol-containing polymer, (b, c) immobilization of azide-containing molecules, and (d, e) specific binding of targeted molecules [55].

Thanks to the anti-biofouling property of zwitterions, the immune system's detection of the zwitterionic polymer-based drugs and the circulation time of the designed nanocarriers is prolonged, which can accumulate in solid tumors with the help of enhanced permeability and retention effect, a phenomenon that enables the increased tumor accumulation of drugs and proteins that are attached to macromolecular carriers [57]. Drug delivery is the area in which the usage of zwitterionic polymers has advantages on PEG-based systems because of the sensitivity of PEGs to oxidation and deactivation at temperatures above 35 °C. In

contrast, thermally and chemically stable zwitterionic polymers do not reduce protein activity.

Cancer cells have incredibly high amounts of negatively charged glycoproteins on their external surface, which operate as an electrical shield [58]. Using a positively charged nanoparticle in drug delivery systems will improve cellular internalization. Because of the pH difference between regular (pH 7.2–7.4) and tumor tissue (pH 6.0–6.8), targeted nanoparticle accumulation in tumor tissues for cancer treatment and/or imaging is an interesting method. Because increasing the medium's acidity causes a divergence from the isoelectronic point, the pH dependency of zwitterions can be employed for this purpose. The surface charge of the polymers will switch from negative or neutral status to positive if the polymer has pH-sensitive bonds. Because of their higher affinity for negatively charged cell membranes, the positively charged nanoparticles can easily enter cells and induce cellular internalization [59]. Charge-reversal micelles are potential systems to serve this methodology. Using controlled-ring opening polymerization, Wang et al. created a charge-reversal micelle system from the block copolymers poly(-caprolactone) (PCL) and poly(allyl ethylene phosphate) (PCLb-PAEP). The reaction of cysteamine hydrochloride (Cya) and 2-(mercaptoethyl) trimethylammonium chloride by thiol-ene “click” chemistry, followed by the reaction with 2,3-dimethylmaleic anhydride, results in the polymer PCL-b-P(AEPg-TMA/DMA) as shown in the Figure 1.15 [60].

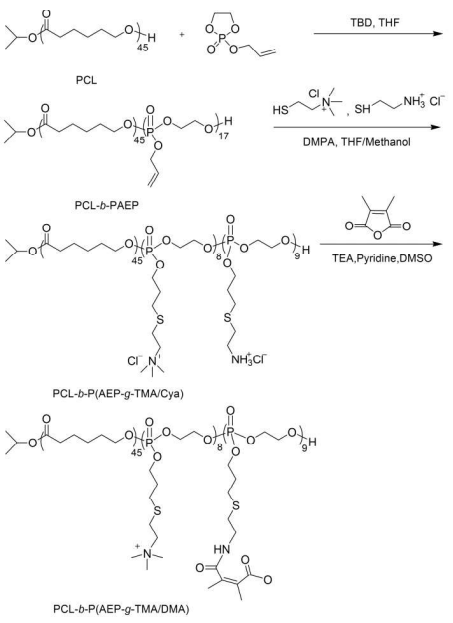

Figure 1.15. Synthesis of PCL-b-P(AEPg-TMA/DMA [61].

In aqueous solutions, PCL- b -P(AEPg -TMA/DMA self-assembles to create doxorubicin (DOX)-loaded zwitterionic polymer-based nanoparticles. The zwitterionic polymer decreases its anionic component in reaction to the tumor's acidity, producing PCL-b-P(AEP-g-TMA/Cya). Tumor cells identify the resultant nanoparticles when they are activated to become positively charged (Figure 1.16) [60]. However, carboxyl amide bond cleavage can occur even at neutral pH and undergo hydrolysis in blood at pH=7.4, which causes the premature release of the drug and increase in toxicity. Protonation is an excellent alternative to making polymers positively charged to overcome this problem. At tumor pH, alkoxyphenyl acylsulfonamide ligands become cationic by protonation, increasing cell uptake and cytotoxicity (Figure 1.17) [61].

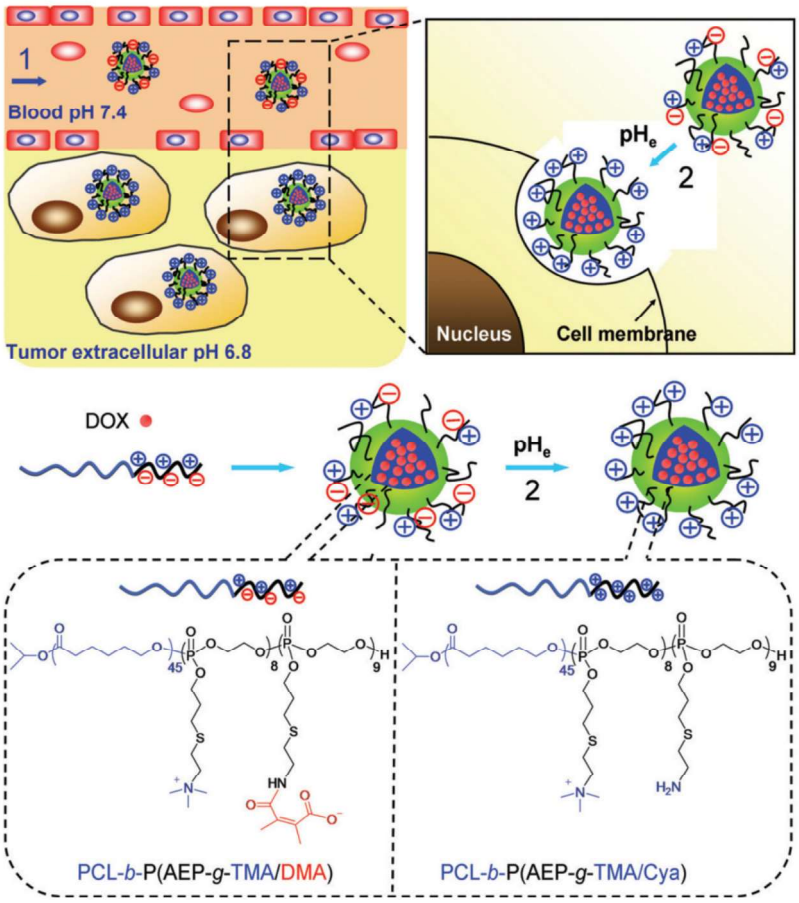

Figure 1.16. Schematic illustration of how the tumor acidity (pH_e) affects the surface charge properties [60].

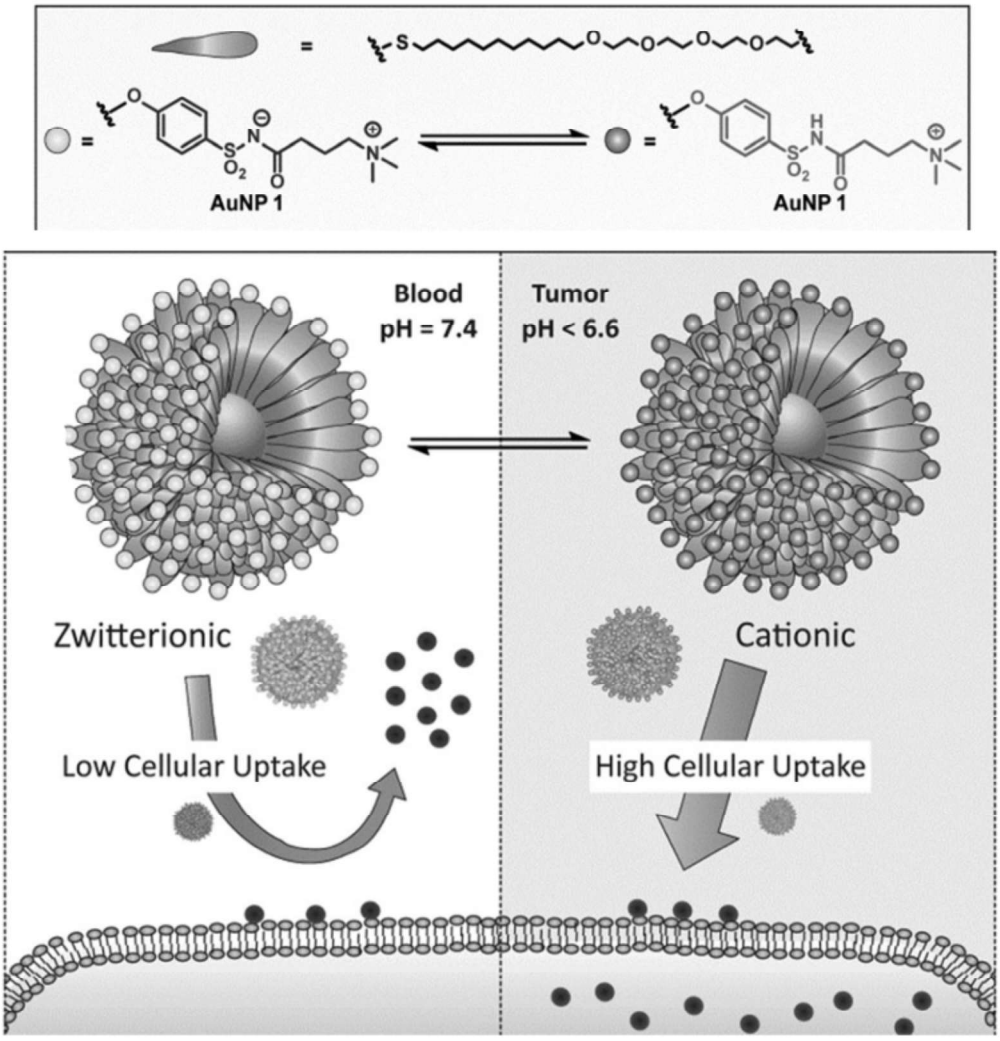

Figure 1.17. Monolayer-protected gold nanoparticles and their pH-responsive delivery strategy [61].

Xiang, T. et al. used a technique for introducing zwitterionic poly(sulfobetaine methacrylate) (PSBMA) by combining ATRP and click chemistry to create blood-contacting membranes with effective blood compatibility with an enhanced antifouling. At first, ATRP was used to develop the SBMA polymers that were functionalized with alkynyl groups. Then, click chemistry was used to graft the functional polymers onto the azido-functionalized membrane surfaces. The membranes with zwitterionic polymer (PSBMA) grafts showed good resistance to bacterial adhesion and protein adsorption (Figure 1.18) [62].

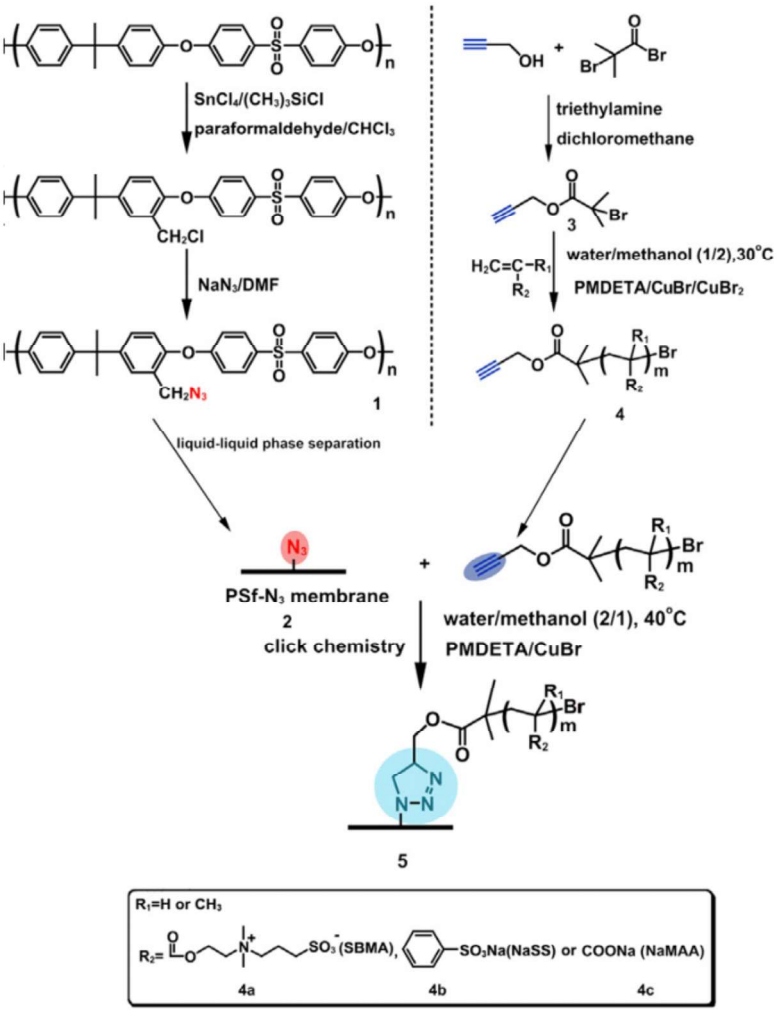

Figure 1.18. Diagram showing the click chemistry-based synthesis of the polymers and grafted membranes [62].

Because of the restrictive reaction conditions or time-consuming procedures needed for surface modification, the controlled construction of bifunctional polymers on surfaces is particularly challenging. Xu, G. et al, devised a quick and efficient approach for attaching cationic and zwitterionic binary polymer brushes to stainless steel (SS) surfaces with polydopamine anchors. Through a thiol-ene "click" reaction, zwitterionic poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) was first grafted from the functionalized SS surface. Then, by an azide-alkyne "click" reaction, alkynyl-modified cationic poly(2-(methacryloyloxy) ethyl trimethylammonium chloride) (alkynyl-PMETA) was added. Thus, a different method for creating surface coatings with multi functionalities was developed (Figure 1.19) [63].

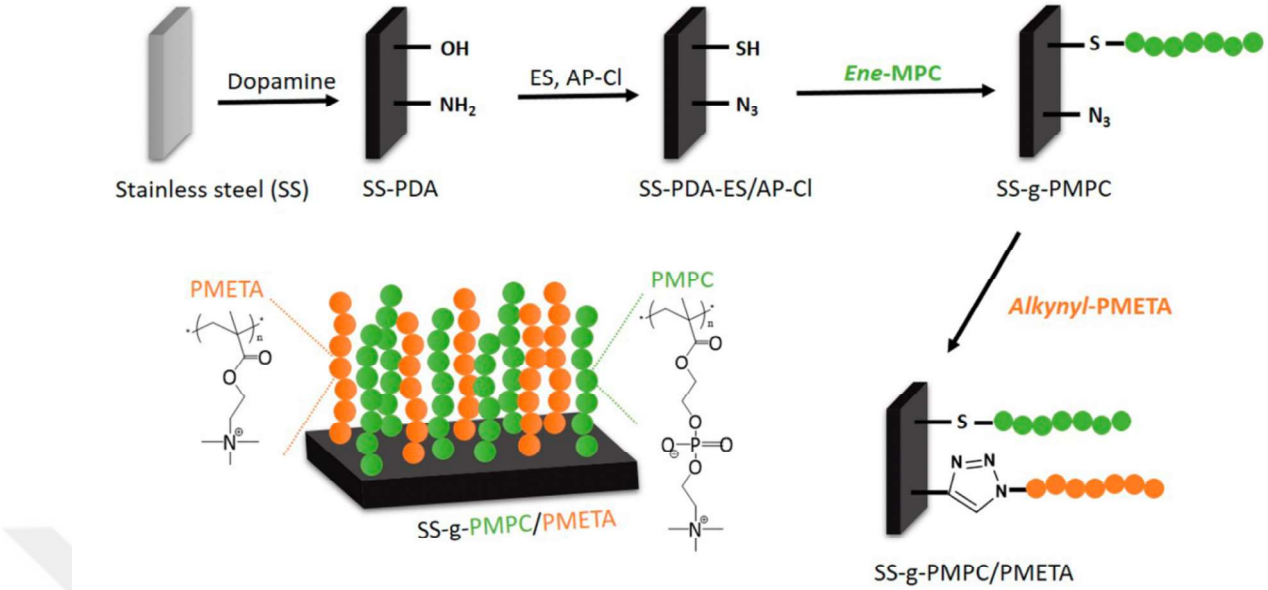

Figure 1.19. Diagrammatic Illustration of "Click" Reaction-Based Fabrication of Binary Polymer Brush Coatings on Stainless Steel Surface [63].

1.4. Diels-Alder Chemistry

As explained above, many application areas of zwitterionic polymers are modified by different functional groups. As a powerful click reaction, the Diels-Alder chemistry has not been used with zwitterionic polymers to design functional polymers that can be used in drug delivery or protein isolatiıon. In Diels-Alder chemistry, a diene, an electron-deficient molecule, and a dienophile, an electron-rich molecule, perform a [4+2]-cycloaddition process utilizing their 4π and 2π electrons, respectively, to produce a cyclohexene ring in a single step in the DA reaction (Figure 1.20). The stability of newly formed σ-bonds is the energetically more stable bonds than π-bonds, which drives this process. It is a one-step thermoresponsive and biocompatible "click" reaction that does not require a catalyst and produces no side products [64,65].

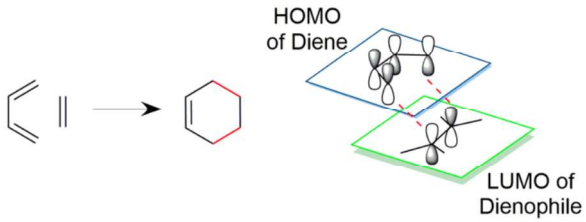

Figure 1.20. The DA reaction between a diene and a dienophile [66].

Generally, furan and anthracene groups have been employed in macromolecular synthesis as diene components. The maleimide moiety is a common dienophile because of its strong reactivity and structural diversity. It is feasible to shift the reaction towards the reactants in the furan-maleimide cycloadduct by simply heating to high temperatures, through the retro Diels-Alder reaction (rDA) (Figure 1.21) [67].

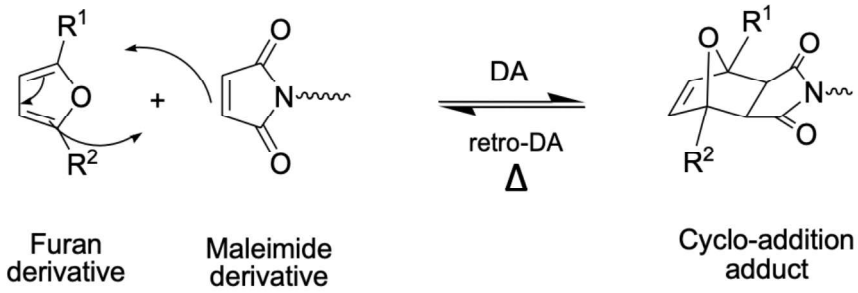

Figure 1.21. Diels-Alder and Retro Diels-Alder reactions.

The 1,4- Michael addition reaction, which relies on the nucleophilic attack of an electron-rich thiol on an electron-deficient N-substituted maleimide with two electron-withdrawing groups, is a classic example of the nucleophilic thiol-ene reaction. The absence of additional reagents and the lack of side reactions make this procedure particularly appealing for modifying proteins and peptides. Due to steric hindrance or solubility issues, moderate bases like triethylamine may be required to start the reaction. This addition process is ideal for biomedical applications because it does not require any metal-based catalysts (Figure 1.22) [68, 69].

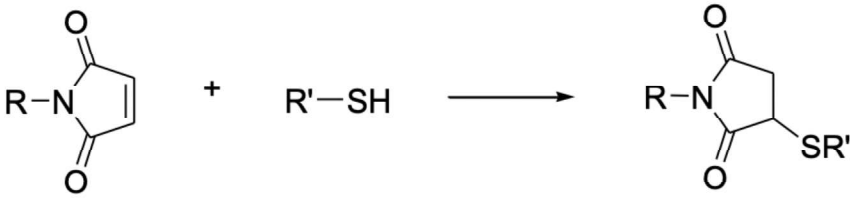

Figure 1.22. A representative nucleophilic Michael addition thiol-ene reaction.

For use as targeted drug delivery systems, the Sanyal and coworkers reported PEG-based copolymers that can be functionalized with thiol-containing molecules. Polymers with side chains reactive towards thiol-containing molecules are obtained using a monomer containing a furan-protected maleimide group. The retro Diels-Alder reaction was then used to activate the maleimide groups, enabling the polymer to be conjugated with thiol-containing hydrophobic dyes such as BODIPY-SH, and a cell targeting group such as cyclic peptide cRGDfC. It was evident that the addition of the peptide-based targeting group improved the cellular internalization of the dye conjugated polymer (Figure 1.23) [70].

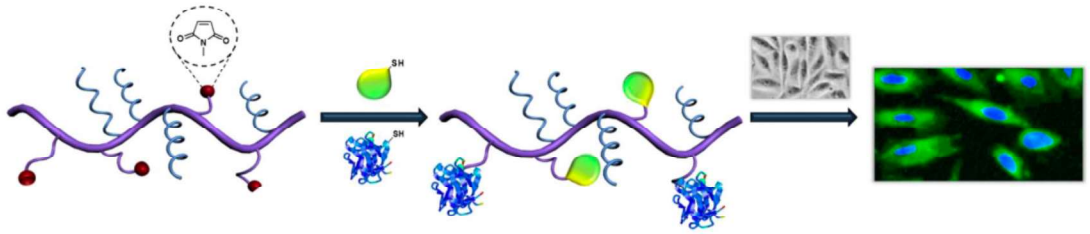

Figure 1.23. Cellular targeting and multifunctionalization of copolymers with thiol reactions that contain maleimides [70].

Another study that uses the strategy-making nanogels by the reaction of thiols and the double-bonds of furan-maleimide moieties was reported by Phan, Q. T. et al. [71] They synthesized a nanogel via a one-pot click reaction of sulfobetaine and cystamine via atom transfer radical polymerization with furan protected maleimide adduct functionalized initiators. After azide-functionalized polysulfobetaine is obtained, the thiolactone derivative is conjugated to the polymer. Nanogels were obtained using a diamine crosslinker under UV radiation, which opened the lactone rings and released thiol groups that reacted with the double-bonds of furan-maleimide moieties. The crosslinker contains a degradable group,

which enables the release of the cancer drug in the presence of glutathione (Figure 1.24) [71].

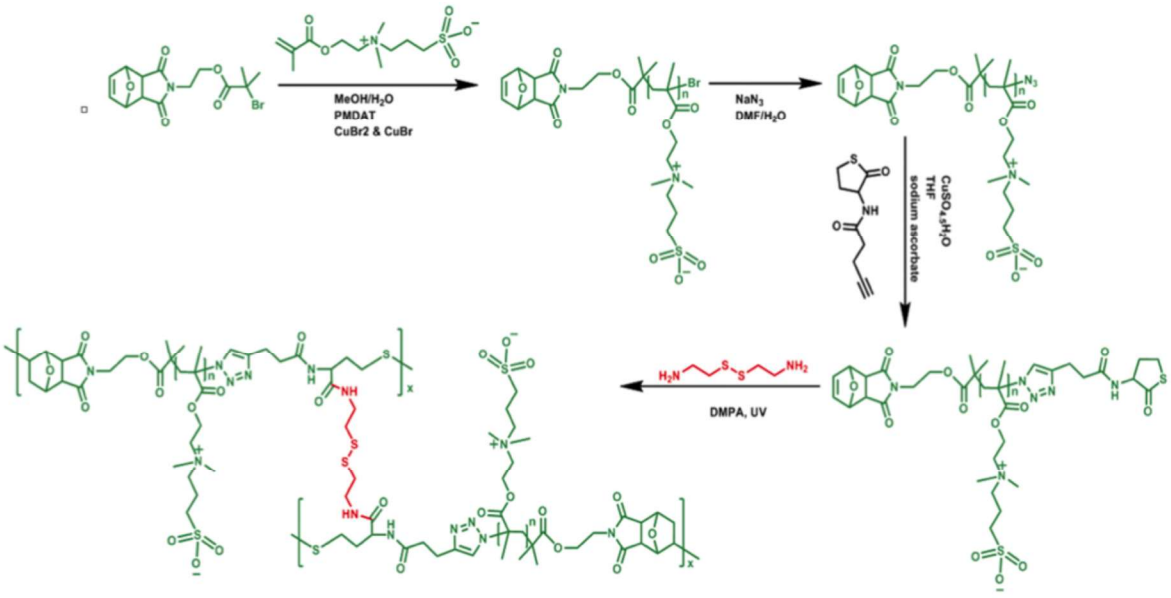

Figure 1.24. Redox-responsive nanogel synthesis [71].

Becker, G. et al. worked on thermoresponsive polymers since those materials are studied for use in sensor systems and drug delivery, among many other applications. They provided reversible post-polymerization modification that allows degradable poly(phosphester) (PPE) copolymers with lower critical solution temperature to be further adjusted. The solubility profile of the polymers was modified using the Diels-Alder reaction with maleimides of various hydrophilicity, in which zwitterionic structures (V) were used to obtain complete solubility at basic or acidic conditions (Figure 1.25) [72].

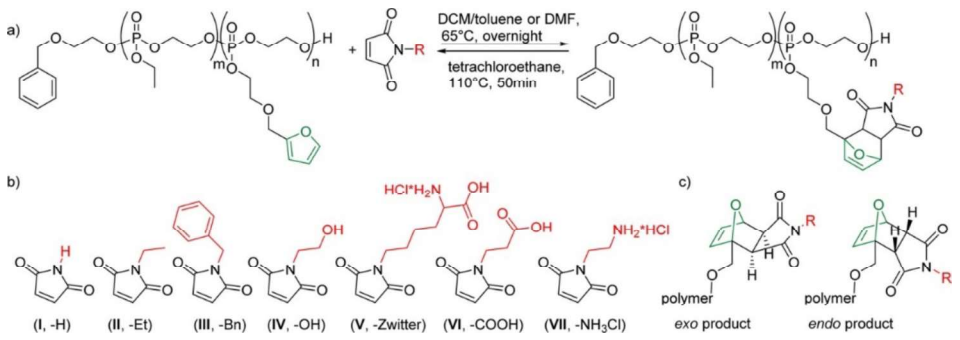

Figure 1.25. Diels–Alder reaction between furfuryl-containing polymers and maleimide-containing structures (V) [72].

2. AIM OF THE STUDY

This study aims to synthesize thiol-reactive zwitterionic polymeric structures containing thiol-reactive maleimide entities as side chains. The zwitterionic nature of the polymer was chosen to design a system as an alternative to PEG-based polymers. Maleimide-containing zwitterionic polymers with "reactive" functional groups will be easily functionalized by "click" chemistry with thiol-containing compounds. A protection-deprotection approach was employed to preserve the reactive maleimide double bond. By using the retro Diels-Alder reaction, the maleimide groups in the side chains of the polymers were converted into their reactive state. The extent of the retro Diels-Alder reaction can be controlled by using different reaction periods. Thus, a polymer containing two orthogonally reactive groups will be obtained: the thiol-reactive maleimide group and the oxanorbornene alkene-containing bicyclic group. The latter group can be functionalized with radical thiol-ene and inverse-electron-demand Diels-Alder reactions. One can envision that the norbornene alkene groups in the polymer could be conjugated with cell targeting groups using other click-type conjugations to enable cell imaging.

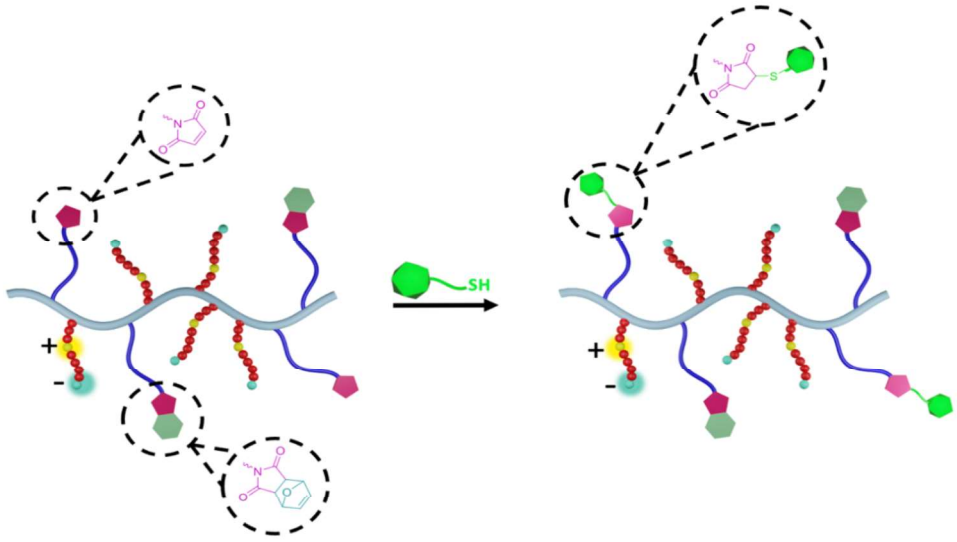

Figure 2.1. Scheme of thiol-reactive maleimide-containing zwitterionic copolymers.

3. EXPERIMENTAL

3.1.1. Materials

Maleic anhydride, methanol (MeOH), chloroform ($CDCl_3$), triethylamine (TEA), 4-(Dimethylaminno)pyridine (DMAP), and N-(3-Sulfopropyl)-N-methacroyl-oxyethyl-N,N-dimethylammonium betaine (SBMA) were purchased from Merck. Furan (>99%), 2,2,2-Trifluoroethanol (>99%) (TFE) were purchased from Alfa Aesar. 3-amino-1-propanol, methacrylic anhydride, 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid (CTA), and 4,4'-Azobis(4-cyanovaleric acid) (ACVA) were purchased from Sigma-Aldrich. Anhydrous solvents such as CH_2Cl_2, tetrahydrofuran (THF), and toluene was obtained from ScimatCo Purification System. Aysun Değirmenci prepared BODIPY-SH according to literature procedures [73-75]. The dialysis bags (Spectra/Por® Biotech Regenerated Cellulose Dialysis Membranes, MWCO 12000-15000 Da) were purchased from Spectrum Labs.

3.1.2. Instrumentation

Synthesized monomer and polymer characterizations were carried out with ^1H NMR spectroscopy (Varian 400 MHz) at 25 °C. The measurements were performed in deuterated chloroform ($CDCl_3$) and water (D_2O). Fourier transform infrared (FT-IR) spectroscopy as Thermo Fisher Scientific Inc. Nicolet 380 with 7800 – 350 cm^{-1} using proprietary KBr beamsplitter. The UV spectrum was measured with a Varian Cary-100 UV-Vis Spectrophotometer, centrally controlled by a single dispersion equipped with a high-performance R928 photomultiplier tube. At room temperature, the Varian Cary Eclipse Spectrophotometer was used to measure the fluorescence spectrum. The rectangular quartz cuvette has a 1 cm route length.

3.1.3. Synthesis of furan-maleimide Monomer

A three-step synthesis route was followed to synthesize the furan-maleimide monomer according to the literature procedure[76] with some modifications. Briefly, in the first step, maleimide was protected by furan to synthesize furan-maleimide adduct. The

reaction was held in toluene at 80 °C for 6 hours under reflux. Second, alcohol exo-3a,4,7,7a-tetrahydro-2-(3-hydroxypropyl)-4,7-epoxy-1H-isoindole-1,3(2H) dione was obtained by the reaction of the purified furan-maleimide adduct with 3-amino-1-propanol at 80 °C. The reaction was left stirring overnight under reflux. Lastly, purified furan-protected maleimide containing alcohol was reacted with methacrylic anhydride at room temperature to obtain the furan-masked maleimide monomer. Products of all three steps were characterized using ^{1}H-NMR.

For cycloadduct: ^{1}H NMR ($CDCl_3$, δ, ppm) 6.54 (s, 2H, C*H*=C*H*), 5.42 (s, 2H, C*H* bridgehead protons), 3.15 (s, 2H, C*H*-C*H*, bridge protons).

For alcohol: ^{1}H NMR ($CDCl_3$, δ, ppm) 6.52 (s, 2H, C*H*=C*H*), 5.26 (s, 2H, C*H* bridgehead protons), 3.64 (t, 2H, J=6.2 Hz, OCH_2), 3.51 (t, 2H, NCH_2), 2.87 (s, 2H, C*H*-C*H*, bridge protons), 2.29 (s, 1H, O*H*), 1.76 (m, 2H, $CH_2CH_2CH_2$).

For furan-masked maleimide monomer: ^{1}H NMR ($CDCl_3$, δ, ppm) 6.51 (s, 2H, C*H*=C*H*), 6.13 (s, 1H, CH_2=C), 5.57 (m, 1H, CH_2=C), 5.26 (s, 2H, C*H* bridgehead protons), 4.10 (t, 2*H*, J=6.2 Hz, OCH_2), 3.61 (t, 2H, J=7.0 Hz, NCH_2), 2.84 (s, 2H, C*H*-C*H*, bridge protons), 1.95 (m, 5H, $CH_2CH_2CH_2$, CH_3).

3.1.4. Copolymerization of the Latent Reactive Monomer and SBMA

3.1.4.1. Synthesis of Copolymer **P1**: In a typical copolymerization (for **P1** copolymer), furan protected maleimide monomer **(3)** (13.75 mg, 0.047 mmol), SBMA (250 mg 0.895 mmol), the RAFT chain transfer agent, CTA (3.34 mg, 0.012 mmol), and the azo initiator, ACVA (0.70 mg, 0.0025 mmol) were dissolved in 1 mL TFE and was deoxygenated under N_2 atmosphere for 20 minutes. The reaction mixture was stirred at 70 °C for 2 hours. The reaction was purified by dialysis over distilled water using a 12000-15000 Da cut-off cellulose-generated membrane, and water was removed with the help of a lyophilizer. **P1** (202,3 mg) was obtained as a pink powder solid. [SBMA]:[**3**] = 19:1, conversion = 77 %, $M_{n,NMR}$ = 63579 g/mol.

For polymer **P1**: ^{1}H NMR (400 MHz, D_2O, δ, ppm) 8.05-7.77-7.58 (br s, 5H, C_6H_5), 6.58 (s, 2H, C*H*=C*H*), 5.31 (s, 2H, C*H* bridgehead protons), 4.54 (br s, 2H, OCH_2 ester protons of SBMA), 3.85 (br s, 2H, CH_2CH_2N protons of SBMA), 3.63 (br s, 4H, NCH_2CH_2 of SBMA and CH_2CH_2N of **3**), 3.27 (s, 6H, CH_3NCH_3), 3.02 (s, 4H, C*H*-C*H*, bridge protons, and SCH_2 of SBMA), 2.31 (s, 2H, $CH_2CH_2CH_2$ protons of SBMA), 2.01-1.02 (m, CH_3, and CH_2 along polymer backbone).

3.1.4.2. Synthesis of Copolymer **P2**: Furan protected maleimide monomer **(3)** (28.9 mg, 0.099 mmol), SBMA (250 mg 0.895 mmol), the RAFT chain transfer agent, CTA (3.34 mg, 0.012 mmol), and the azo initiator, ACVA (0.70 mg, 0.0025 mmol) were dissolved in 1 mL TFE and was deoxygenated under N_2 atmosphere for 20 minutes. The reaction mixture was stirred at 70 °C for 2 hours. The reaction was purified by dialysis over distilled water using a 12000-15000 Da cut-off cellulose-generated membrane, and water was removed with the help of a lyophilizer. **P2** (201.7 mg) was obtained as a pink powder solid. [SBMA]:[**3**] = 9:1, conversion = 76 %, $M_{n,NMR}$ = 82198 g/mol.

For polymer **P2**: ^{1}H NMR (400 MHz, D_2O, δ, ppm) 8.05-7.77-7.58 (br s, 5H, C_6H_5), 6.58 (s, 2H, C*H*=C*H*), 5.31 (s, 2H, C*H* bridgehead protons), 4.54 (br s, 2H, OCH_2 ester protons of SBMA), 3.85 (br s, 2H, CH_2CH_2N protons of SBMA), 3.63 (br s, 4H, NCH_2CH_2 of SBMA and CH_2CH_2N of **3**), 3.27 (s, 6H, CH_3NCH_3), 3.02 (s, 4H, C*H*-C*H*, bridge protons, and SCH_2 of SBMA), 2.31 (s, 2H, $CH_2CH_2CH_2$ protons of SBMA), 2.01-1.02 (m, CH_3, and CH_2 along polymer backbone).

3.1.4.3. Synthesis of Copolymer **P3**: Furan protected maleimide monomer **(3)** (65 mg, 0.224 mmol), SBMA (250 mg, 0.895 mmol), the RAFT chain transfer agent, CTA (3.34 mg, 0.012 mmol), and the azo initiator, ACVA (0.70 mg, 0.0025 mmol) were dissolved in 1 mL TFE and was deoxygenated under N_2 atmosphere for 20 minutes. The reaction mixture was stirred at 70 °C for 2 hours. The reaction was purified by dialysis over distilled water using a 12000-15000 Da cut-off cellulose-generated membrane, and water was removed with the help of a lyophilizer. **P3** (210,5 mg) was obtained as a pink powder solid. [SBMA]:[**3**] = 4:1, conversion = 55 %, $M_{n,NMR}$ = 49105 g/mol.

For polymer **P3**: ^{1}H NMR (400 MHz, D_2O, δ, ppm) 8.05-7.77-7.58 (br s, 5H, C_6H_5), 6.58 (s, 2H, C*H*=C*H*), 5.31 (s, 2H, C*H* bridgehead protons), 4.54 (br s, 2H, OCH_2 ester protons of SBMA), 3.85 (br s, 2H, CH_2CH_2N protons of SBMA), 3.63 (br s, 4H, NCH_2CH_2 of SBMA and CH_2CH_2N of **3**), 3.27 (s, 6H, CH_3NCH_3), 3.02 (s, 4H, C*H*-C*H*, bridge protons, and SCH_2 of SBMA), 2.31 (s, 2H, $CH_2CH_2CH_2$ protons of SBMA), 2.01-1.02 (m, CH_3, and CH_2 along polymer backbone).

3.1.5. Activation of Copolymers by retro-Diels/Alder Reaction

3.1.5.1. Activation of Copolymer Polymer P1 **(P4)**: (20 mg, 0.786 μmol) was put in a round bottom flask and heated at 110 °C under vacuum for 24 hours. ^{1}H NMR analysis indicated a 50% conversion of the oxabicyclic moiety to the maleimide functional group.

For polymer **P4**: ^{1}H NMR (400 MHz, D_2O, δ, ppm) 6.99 (s, 2H, C*H*=C*H*) 6.58 (s, 2H, C*H*=C*H*), 5.31 (s, 2H, C*H* bridgehead protons), 4.54 (br s, 2H, OCH_2 ester protons of SBMA), 3.85 (br s, 2H, CH_2CH_2N protons of SBMA), 3.63 (br s, 4H, NCH_2CH_2 of SBMA and CH_2CH_2N of **3**), 3.27 (s, 6H, CH_3NCH_3), 3.02 (s, 4H, C*H*-C*H*, bridge protons, and SCH_2 of SBMA), 2.31 (s, 2H, $CH_2CH_2CH_2$ protons of SBMA), 2.01-1.02 (m, CH_3, and CH_2 along polymer backbone).

3.1.5.2. Activation of Copolymer Polymer P1 **(P5)**: (10 mg, 0.157 μmol) was put in a round bottom flask and heated at 110 °C under vacuum for five days. ^{1}H NMR analysis indicated a 93 % conversion of the oxabicyclic moiety to the maleimide functional group.

For polymer **P5**: ^{1}H NMR (400 MHz, D_2O, δ, ppm) 6.99 (s, 2H, C*H*=C*H*) 6.58 (s, 2H, C*H*=C*H*), 5.31 (s, 2H, C*H* bridgehead protons), 4.54 (br s, 2H, OCH_2 ester protons of SBMA), 3.85 (br s, 2H, CH_2CH_2N protons of SBMA), 3.63 (br s, 4H, NCH_2CH_2 of SBMA and CH_2CH_2N of **3**), 3.27 (s, 6H, CH_3NCH_3), 3.02 (s, 4H, C*H*-C*H*, bridge protons, and SCH_2 of SBMA), 2.31 (s, 2H, $CH_2CH_2CH_2$ protons of SBMA), 2.01-1.02 (m, CH_3, and CH_2 along polymer backbone).

3.1.6. Conjugation of Polymer P4 with BODIPY-SH via Thiol-ene Reaction

Polymer **P4** (20.0 mg, 0.314 μmol) was dissolved in 0,1 mL TFE. Triethylamine (0.99 μL, 7.14 μmol) was dissolved in 0.05 mL TFE and added to the reaction mixture. BODIPY-SH (3.0 mg, 7.14 μmol) was also dissolved in 0.05 mL TFE and added to the reaction mixture. The reaction was left stirring at room temperature for 24 h. The conjugate was repeatedly washed with methanol to remove the unreacted dye. According to the ^{1}H NMR analysis, 100% of maleimides were conjugated to BODIPY-SH. The resultant polymer was a fluorescent solid **P6** (17 mg, 85% yield).

For polymer **P6:** ^{1}H NMR (400 MHz, D_2O, δ, ppm), 6.58 (s, 2H, C*H*=C*H*), 5.31 (s, 2H, C*H* bridgehead protons), 4.54 (br s, 2H, OCH_2 ester protons of SBMA), 3.85 (br s, 2H, CH_2CH_2N protons of SBMA), 3.63 (br s, 4H, NCH_2CH_2 of SBMA and CH_2CH_2N of **3**), 3.27 (s, 6H, CH_3NCH_3), 3.02 (s, 4H, C*H*-C*H*, bridge protons, and SCH_2 of SBMA), 2.31 (s, 2H, $CH_2CH_2CH_2$ protons of SBMA), 2.01-1.02 (m, CH_3, and CH_2 along polymer backbone).

4. RESULTS AND DISCUSSION

4.1.1. Synthesis and Characterization of Furan-maleimide Monomer

Three steps reaction was performed to obtain a furan-masked maleimide monomer according to the literature [76]. First, using the Diels-Alder (DA) reaction, maleic anhydride was protected by furan (Figure 2.2a). In the next step, the synthesized adduct is converted into alcohol by the reaction with 3-amino-1-propanol (Figure 2.2b). Lastly, the desired methacrylate monomer containing a furan-protected maleimide moiety was obtained as a white solid by reacting the synthesized alcohol with methacrylic anhydride (Figure 2.2c).

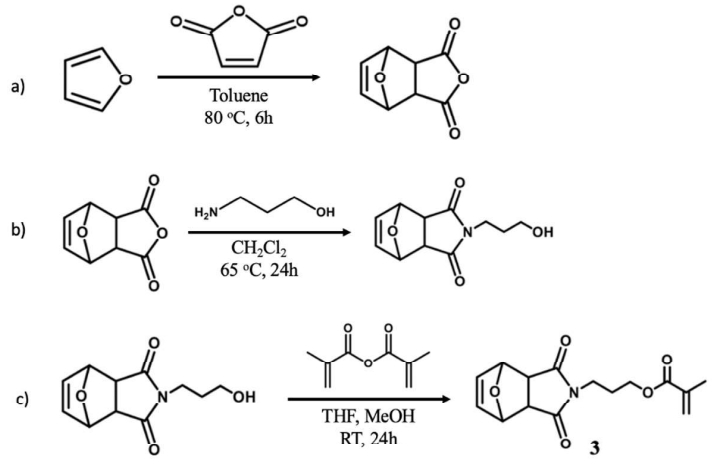

Figure 4.1. Synthesis of the furan-protected maleimide monomer.

In the first step (Figure 2.2a), the adduct was obtained with a yield of 60%. Characterization of the furan-protected maleic anhydride was done by ^{1}H-NMR, and the three peaks from the cycloadduct were seen at 6.54, 5.42, and 3.15 ppm (Figure 2.3). In the synthesis of furan-protected maleimide containing alcohol as a second step, the yield was 62%. ^{1}H-NMR characterization showed four additional peaks at 3.64, 3.51, 1.76, and 2.29 ppm belonging to three -CH_2 groups and -OH group respectively (Figure 2.4). Finally, the furan-protected maleimide containing methyl methacrylate monomer was obtained with a yield of 82%. In ^{1}H-NMR, the peak belonging to the -OH group disappeared, and protons of the methyl group are at 1.95 ppm. Peaks belonging to the vinylic protons on the acrylic component can be observed at 5.55 and 6.11 ppm as resonances (protons g & i) (Figure 2.5).

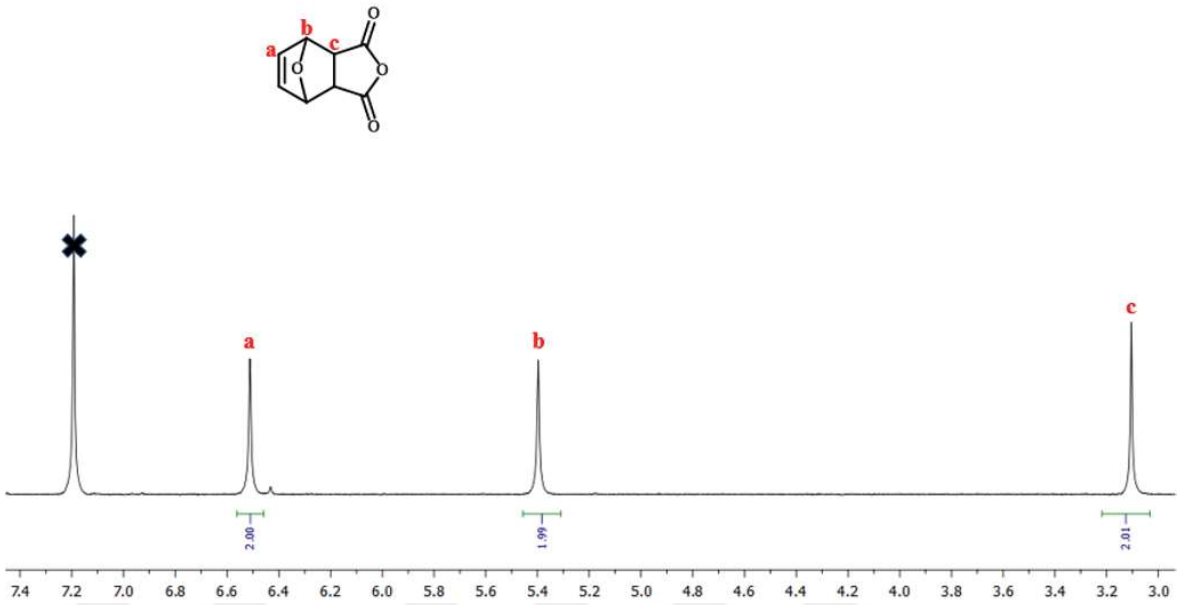

Figure 4.2. ^{1}H-NMR spectrum of furan-protected maleic anhydride in $CDCl_3$.

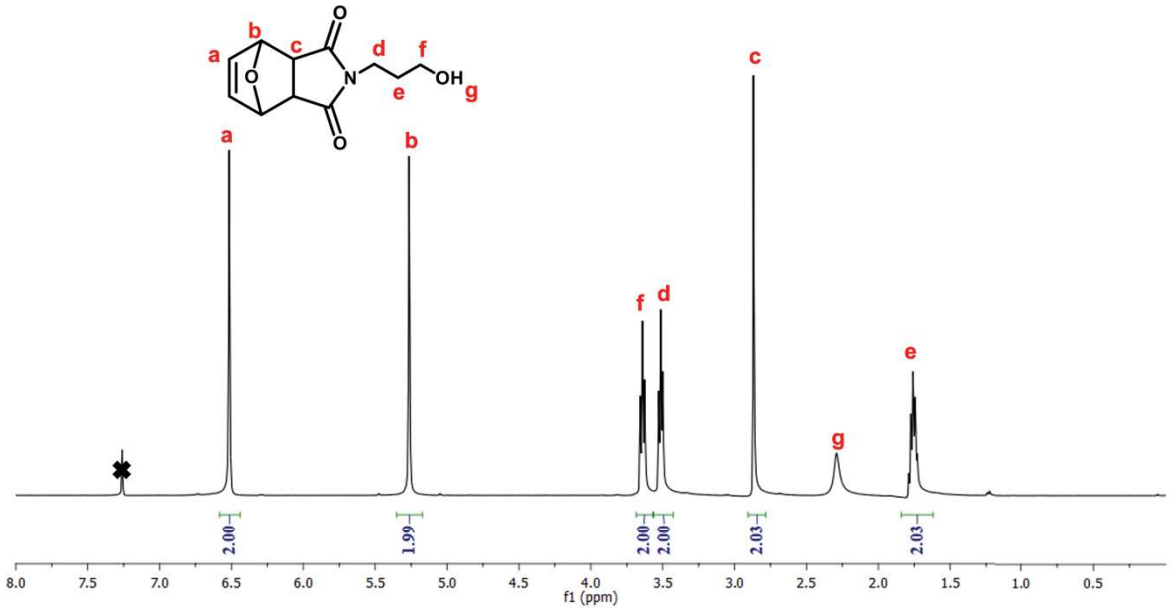

Figure 4.3. ^{1}H-NMR spectrum of furan-protected maleimide containing alcohol in $CDCl_3$.

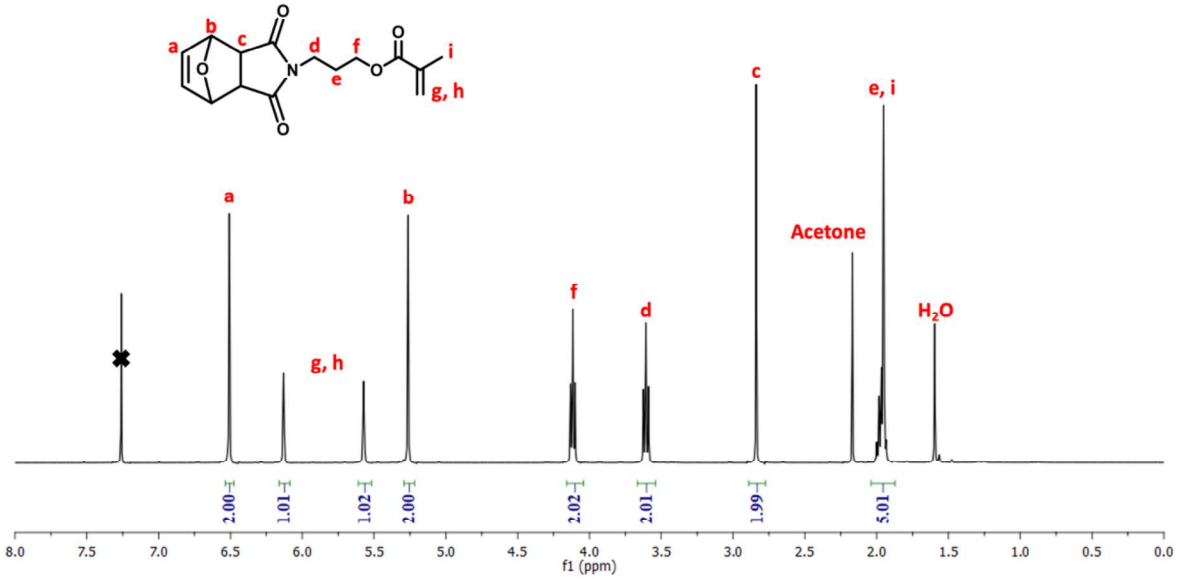

Figure 4.4. ^{1}H-NMR spectrum of furan-protected maleimide containing methyl methacrylate monomer in $CDCl_3$.

4.1.2. Synthesis and Characterization of Zwitterionic Copolymers

The copolymerization of a zwitterionic and a monomer having a maleimide mask forms the reactive polymeric scaffolds mentioned in this thesis. The design of this project was based on the existence of furan-masked maleimide units located at the side chains of the polymer. To produce this architecture, a methacrylate monomer with a furan-protected maleimide group was created in accordance with the literature [76] with minor modifications as explained in the experimental section. Then, this reactive group-containing monomer was copolymerized with a readily available monomer of N-(3-Sulfopropyl)-N-methacroyl-oxyethyl-N,N-dimethylammonium betaine (SBMA) (Figure 2.6).

The monomers that make up the polymeric scaffold play a vital role in their use in numerous bioapplications. Zwitterionic polymeric materials have gained attention thanks to their ultra-hydrophilicity and enhanced anti-biofouling capacity to prevent nonspecific protein adsorption and reduce bacterial or mammalian cell adhesion. In this instance, the zwitterionic SBMA monomer was chosen to design a linear polymeric structure to carry a thiol-reactive functional group at its side chains. Since reversible addition-fragmentation chain transfer (RAFT) polymerization offers narrow polydispersities and high control over molecular weight distribution, it is chosen as the polymerization method. 4-Cyano-4-

(phenylcarbonothioylthio) pentanoic acid (CTA) is selected as the chain transfer agent, and 4,4'-Azobis(4-cyanovaleric acid) (ACVA) is chosen as an azo initiator.

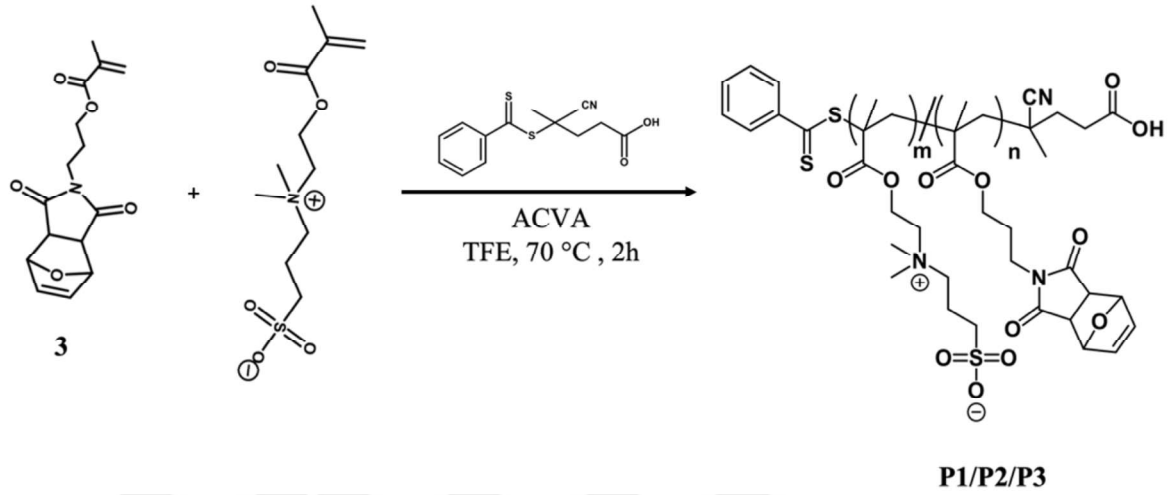

Figure 4.5. Synthesis of the copolymers of latent reactive monomer and SBMA.

The same architecture was used to produce the three copolymers P1, P2, and P3, but with varying amounts of maleimide group content. P3 was designed to have five times as many maleimide groups on its side chain as P1, while P2 was designed to have two times as many. Those three polymeric structures were synthesized by changing the feed ratio of the latent reactive monomer with a constant amount of SBMA. Table 2.1 lists the ratios of the copolymerization reaction's monomers and the characteristics of the generated copolymers.

Table 4.1. Characterization details of copolymers P1, P2, and P3.

No	Polymer	Feed Ratio [M1]:[M2]	Obtained Ratio [M1]:[M2]	M_n NMR (g/mol)	Conversion (%)
1	P1	19:1	19:1	63579	77
2	P2	9:1	9:1	82198	76
3	P3	4:1	4:1	49105	55

M1: SBMA, M2: latent reactive monomer (**3**)

It can be observed on the ^{1}H NMR spectrum of P1 that the integral value of the peak at 6.59 ppm belonging to the cycloadduct of the latent polymer is 22.69, whereas the integral value of the peak at 4.61 ppm belonging to the ester proton of SBMA is 431.55 (Figure 2.7).

From the ratio of those two peaks, one can understand that 5% of the P1 copolymer is the latent reactive monomer. By the same method, P2 and P3 copolymers consist of 10% and 25% of the latent reactive monomer on their structures, respectively, which were the targeted rates (Figures 2.8-2.9).

Additionally, the presence of protons in the C_6H_5 group of CTA can be used to estimate the molecular weight of the polymers since NMR can tell the difference between its proton signals and those of the repetitive monomer group. It is found from the ^{1}H-NMR spectroscopy that P1 copolymer has a molecular weight of 63579 g/mol, whereas P2 and P3 have a molecular weight of 82198 g/mol and 49105 g/mol respectively.

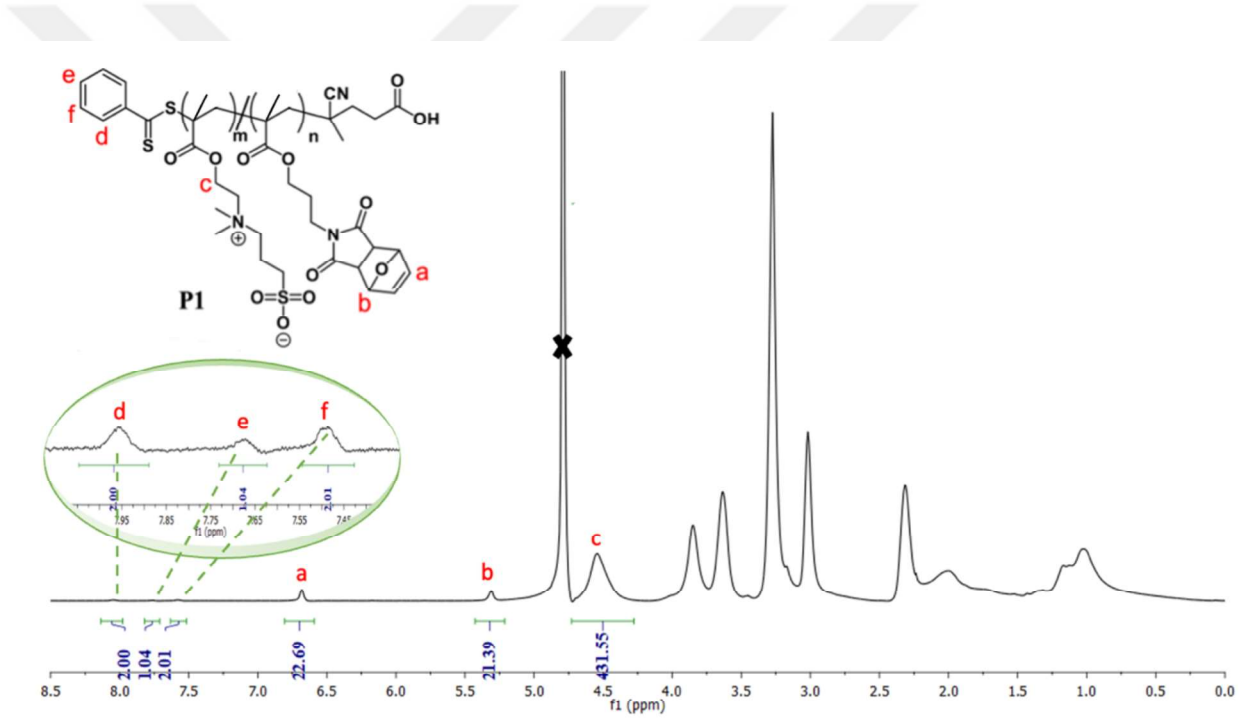

Figure 4.6. ^{1}H-NMR of the copolymer (**P1**) in D_2O.

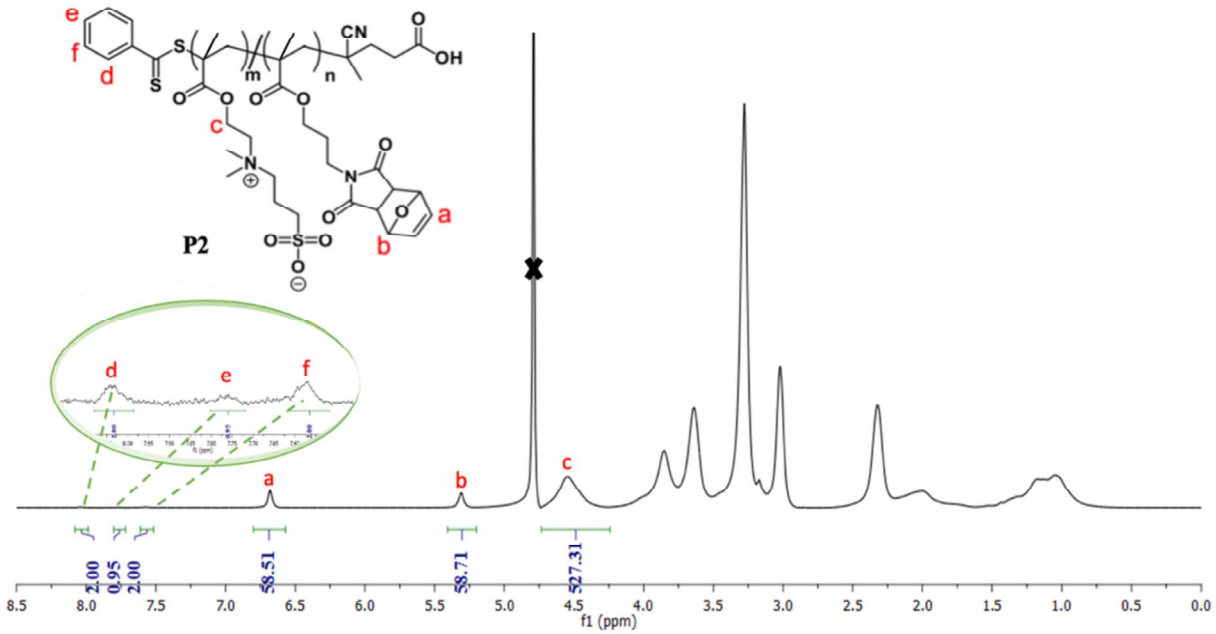

Figure 4.7. ^{1}H-NMR of the copolymer (**P2**) in D_2O.

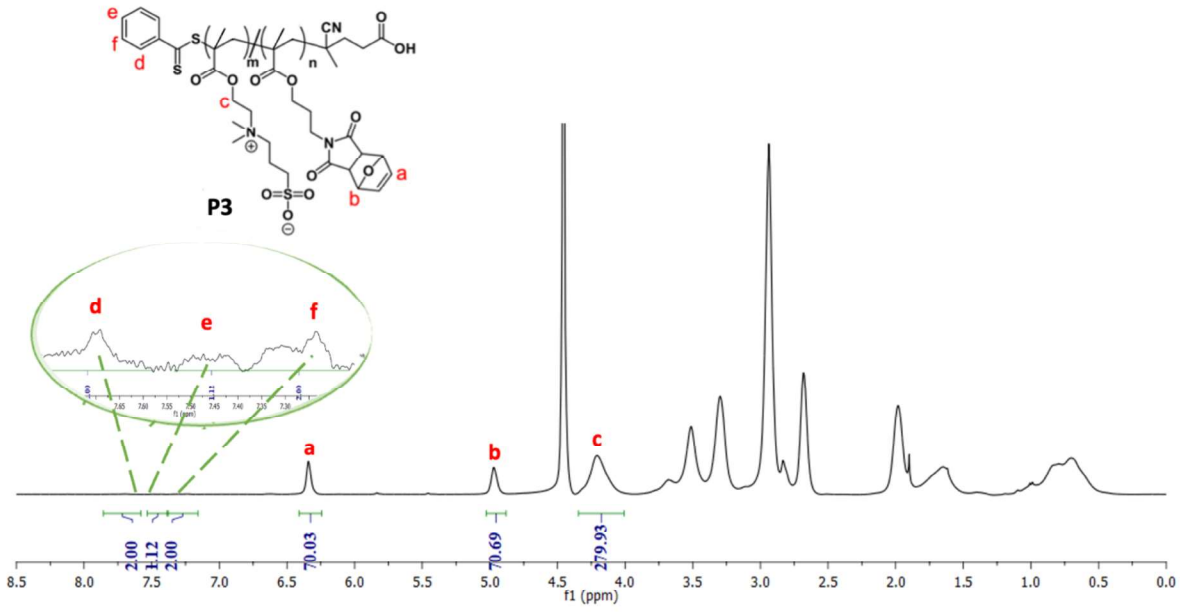

Figure 4.8. ^{1}H-NMR of the copolymer (**P3**) in D_2O.

4.1.3. Activation of the Cycloadduct by the retro-Diels Alder Reaction

Since the Diels-Alder reaction (DA) between the furan and maleimide units occurs at 80°C and the retro Diels-Alder reaction occurs at 110 °C, the latent reactive monomer containing the furan-maleimide cycloadduct unit depends on the protection/deprotection strategy of the maleimide group which is thermoreversible. Therefore, when a furan is used as a protecting group, the polymerizable double bond of a maleimide group cannot participate in copolymerization at 70 °C, the polymerization reaction temperature, thus preventing the cross-linking of monomers.

The copolymer P1 was heated at 110 °C under vacuum after polymerization to remove furan groups from the polymer via the retro Diels-Alder reaction, exposing the maleimide units in active form (Figure 2.10).

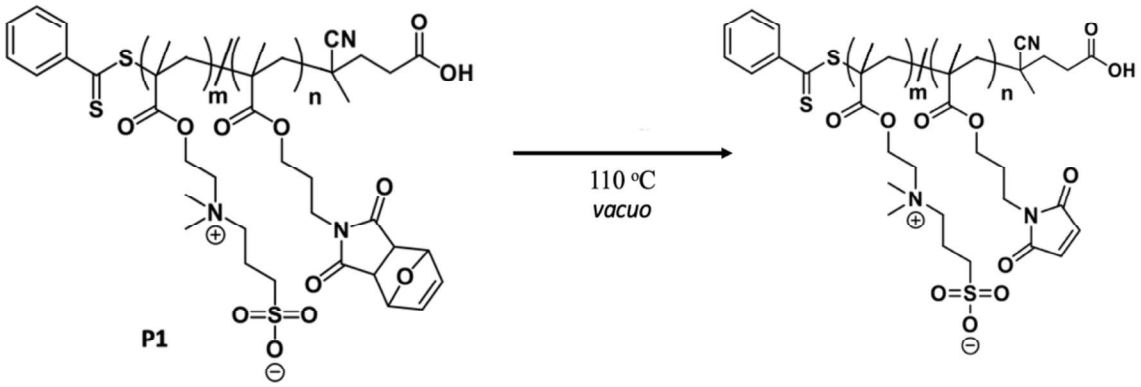

Figure 4.9. Activation of the cycloadduct by the retro DA reaction.

There were two separate retro Diels-Alder experiments with various reaction times. P1 was heated under vacuum for five days to produce copolymer P5, whereas P4 was obtained after a 24-hour reaction time. The appearance of the new peak at 6.99 ppm belonging to the activated maleimide group and decrease in the integrals of the proton resonances belonging to furan groups at 6.59 ppm and 5.22 ppm indicates cycloreversion was partially achieved for both reactions (Figure 2.11).

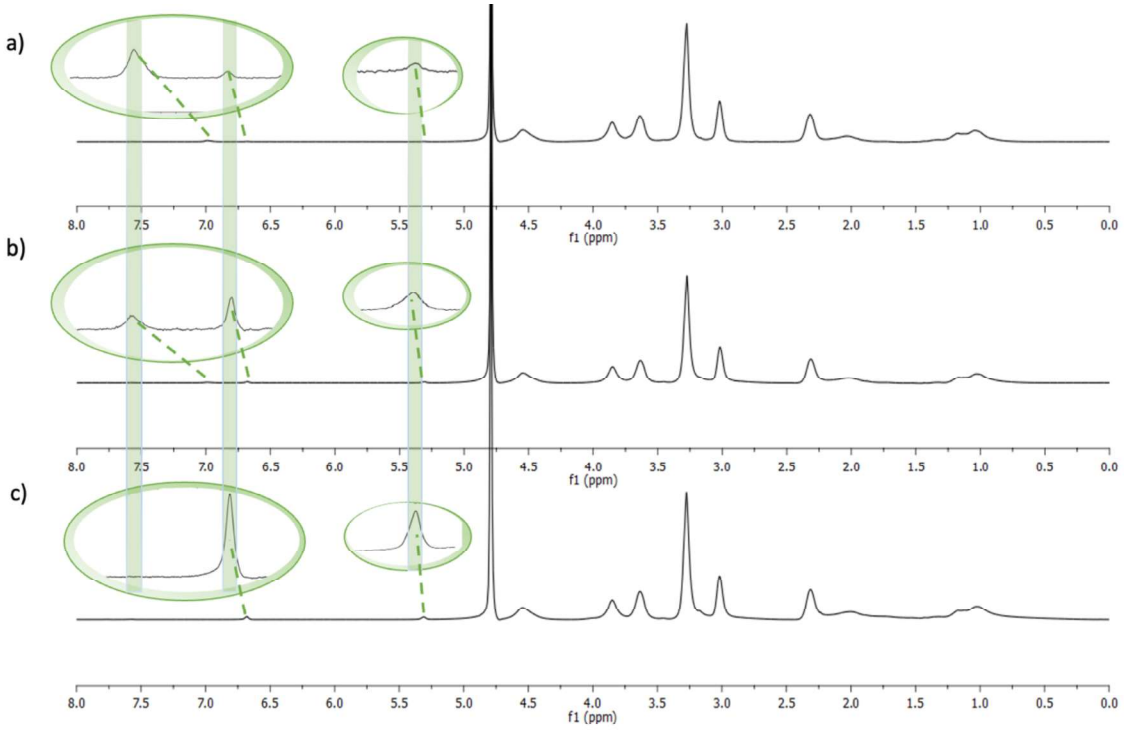

Figure 4.10. ^{1}H-NMR of the (a) copolymer **P5**, (b) copolymer **P4** and (c) copolymer **P1**.

Activated copolymers P4 and P5 were analyzed by ^{1}H NMR spectroscopy to determine the degree of conversion of the cycloadduct to the activated maleimide group and the ratio of monomers after the retro DA reaction. As shown in Figure 2.12, heating the copolymer P1 for 24 hours led to a 50% conversion of the cycloadduct, as determined by taking the ratio of the integrals of the peaks at 4.45 ppm and 6.99 ppm belonging to the newly appeared protons of maleimide group (designated as peak d). The same calculation from the 1H NMR spectrum of P5 shows that heating the P1 copolymer for five days led to 93% conversion (Figure 2.13). Before the retro DA reaction, the integral ratio between the peaks at 4.45 ppm for the ester group of SBMA (designated as peak c) and 6.59 ppm for the unique proton signal of the cycloadduct (designated as peak a) showed that the ratio of the SBMA monomer to non-activated maleimide moiety was 19:1. (Figure 2.7). The ratio for both copolymers, P4 and P5, remained at 19:1 during the retro DA reaction, proved by the same calculations (Figures 2.12 & 2.13).

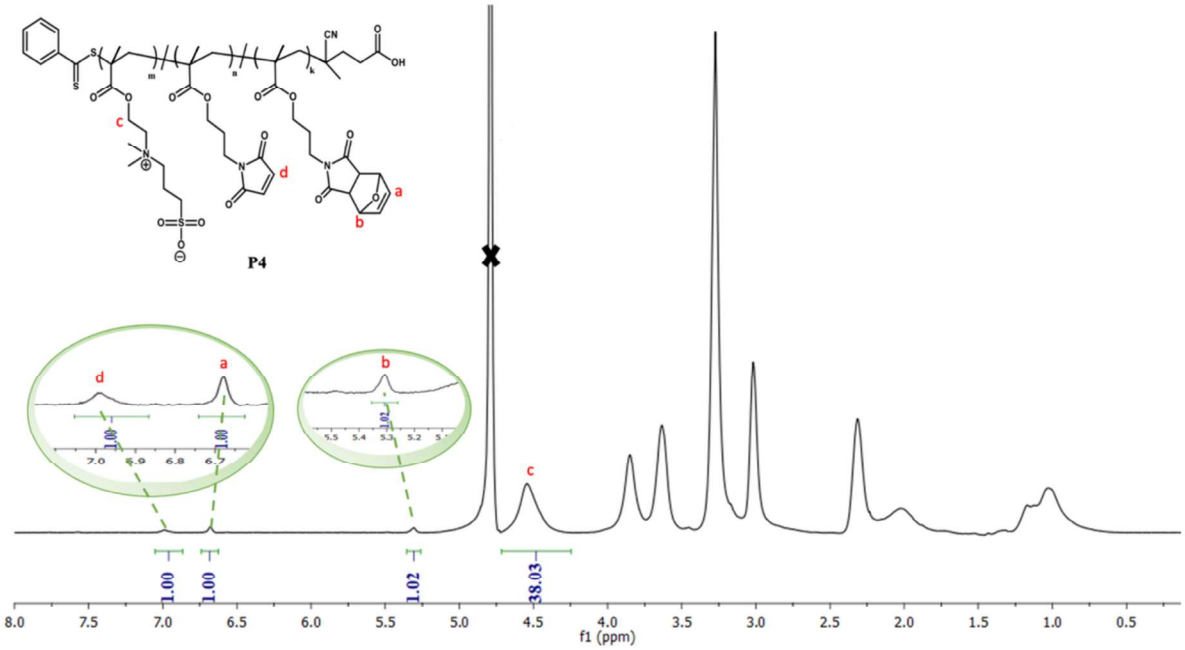

Figure 4.11. ^{1}H-NMR of activated copolymer (**P4**) in D_2O.

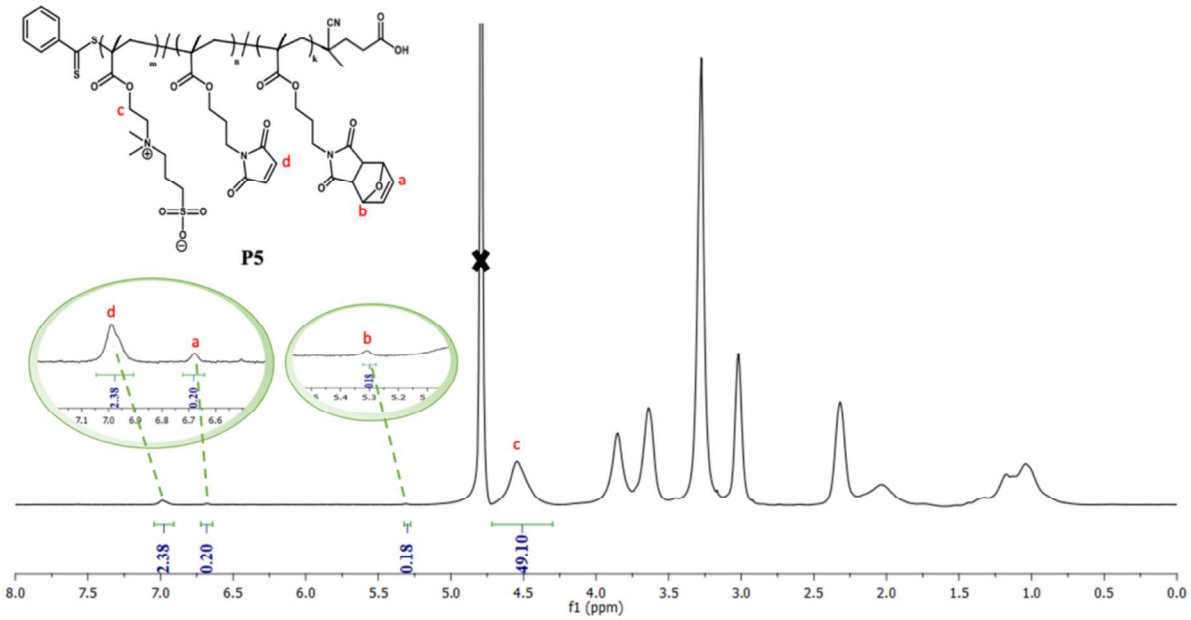

Figure 4.12. ^{1}H-NMR of activated copolymer (**P5**) in D_2O.

FTIR analysis confirmed the effective activation of the polymer's maleimide functional groups by comparing the signals for SBMA homopolymer, copolymers P1, and P5. As a control, a homopolymer of SBMA was synthesized as follows. SBMA (250 mg 0.895 mmol), the RAFT chain transfer agent, CTA (3.34 mg, 0.012 mmol), and the azo

initiator, ACVA (0.70 mg, 0.0025 mmol) were dissolved in 1 mL TFE and were deoxygenated under N_2 atmosphere for 20 minutes. The reaction mixture was stirred at 70 °C for 2 hours. The reaction was purified by dialysis over distilled water using a 12000-15000 Da cut-off cellulose-generated membrane, and water was removed with the help of a lyophilizer. The FTIR spectrum of the resultant homopolymer showed a C═O stretching band belonging to ester groups at ~1721 cm^{-1}. In addition, the asymmetric and symmetric vibrations of the sulfonyl group (SO_3) can be attributed to the bands at ~1170 and ~1034 cm^{-1} (Figure 2.14) [77].

Similarly, copolymers P1 and P5 showed the same vibrations as the homopolymer for the ester and sulfonyl groups. On the other hand, the in-phase C═O stretching vibration of the maleimide unit of the cycloadduct is assigned at ~1694 cm^{-1}, whereas the same vibration belonging to the unmasked maleimide group is at ~1698 cm^{-1} (Figures 2.15 & 2.16). The retro DA reaction shifts the in-phase C═O stretching vibration of the maleimide unit band to a ~4 cm^{-1} higher wavenumber [78].

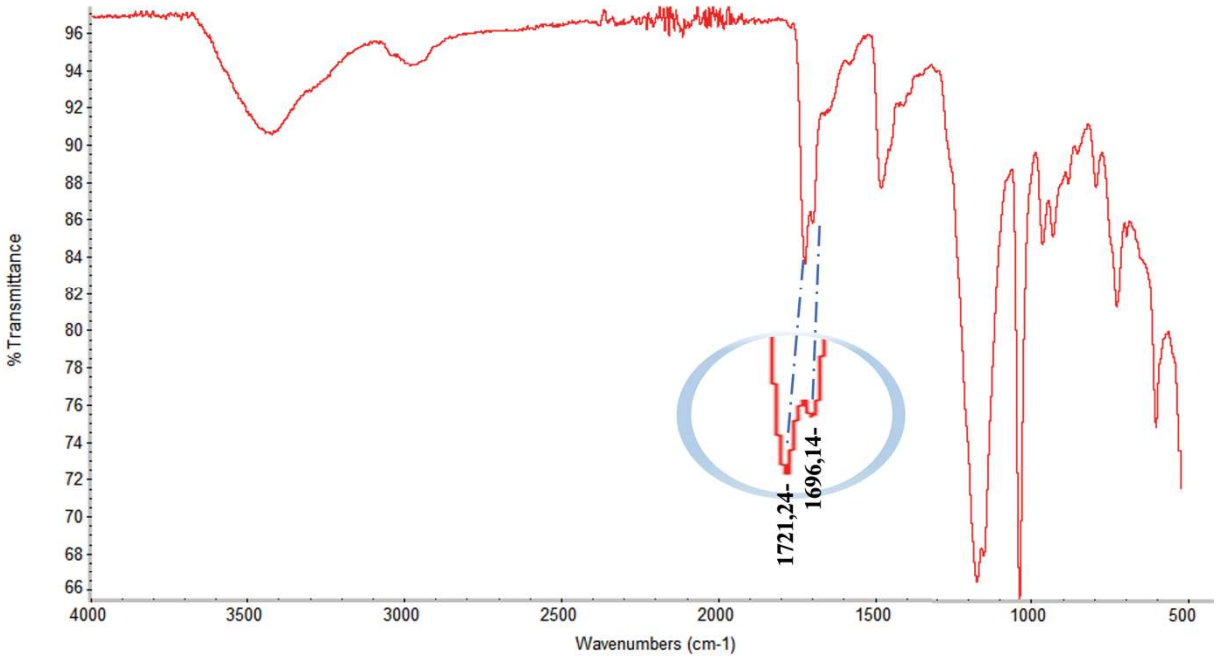

Figure 4.13. FTIR spectrum of the homopolymer of SBMA.

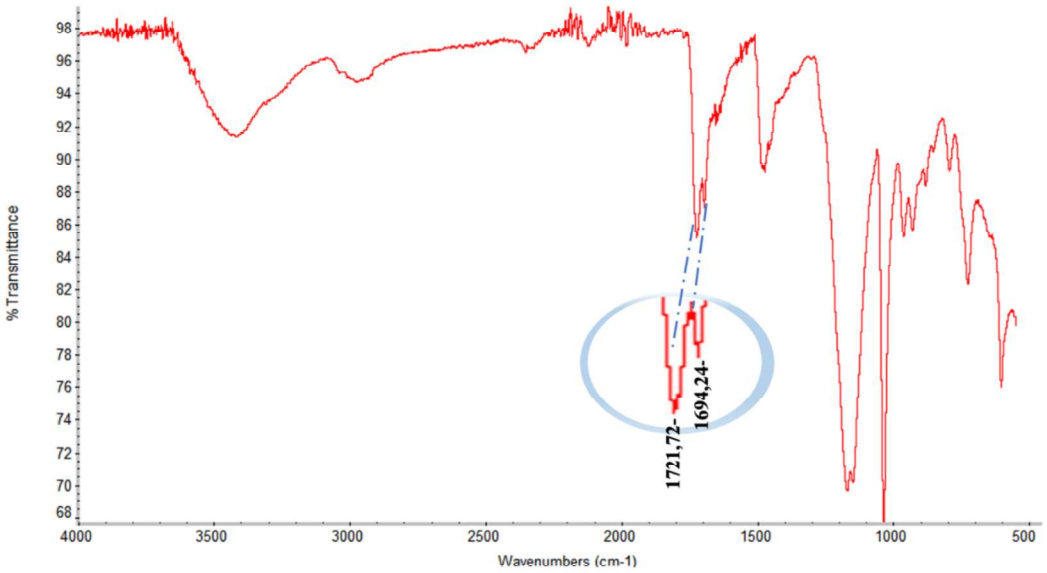

Figure 4.14. FTIR spectrum of copolymer **P1**.

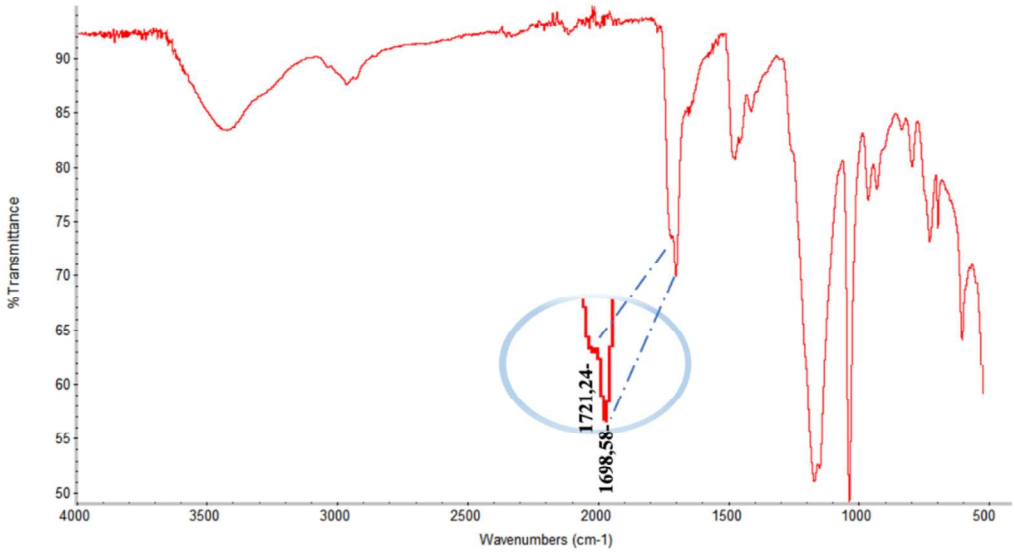

Figure 4.15. FTIR spectrum of copolymer **P5**.

4.1.4. Conjugation of BODIPY-SH to copolymer P4

A fluorescent compound containing a free thiol group was employed for the post-polymerization functionalization after the maleimide units at the side chains of copolymers had been partially activated. The copolymer P4 in which 50% of its maleimide groups were activated was modified by a thiol-containing dye so that a thiol-containing targeting group

can modify the remaining double bonds of the masked-maleimide group under UV irradiation in the future.

Free thiol groups of the hydrophobic dye, BODIPY-SH, enable Michael-type thiol-ene chemistry to conjugate them to copolymers. It is derived from a BODIPY structure with a compound called dipyrromethene complexed with a disubstituted boron atom. By attaching to scaffolds utilized for the delivery of chemotherapeutic drugs or proteins, they can function as imaging agents thanks to the fluorescence feature arising from this structure. Since BODIPY derivates are also recognized for their photodynamic qualities, they also serve as a model of drug conjugation.

Conjugation of the dye to the polymer chains was held in the presence of triethylamine to generate a strong nucleophile of thiol. This reaction was carried out in TFE since the dye is hydrophobic and fluorinated alcohols have a more remarkable ability to dissolve zwitterionic polymers (Figure 2.17). To conjugate BODIPY-SH to all the reactive maleimide groups, a 1:2 ratio of the activated maleimide units to BODIPY-SH dye was used. Aysun Değirmenci synthesized thiol reactive BODIPY-SH dye according to the literature procedures [74].

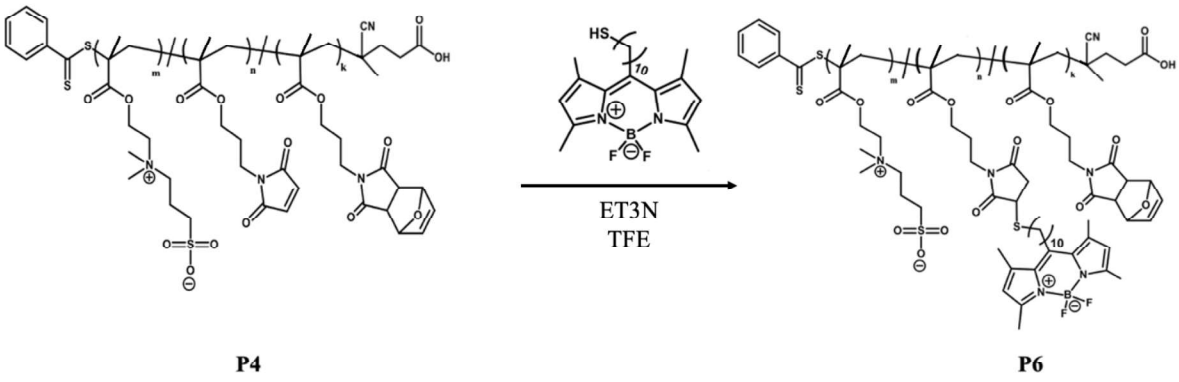

Figure 4.16. BODIPY-SH modification reaction of copolymer **P4**.

Conjugation efficiency was evaluated by removing the activated maleimide group peak on ^{1}H-NMR spectroscopy. As seen in Figure 2.18, the characteristic peak of the proton resonance of the maleimide unit at 6.99 ppm disappeared, proving 100% conjugation.

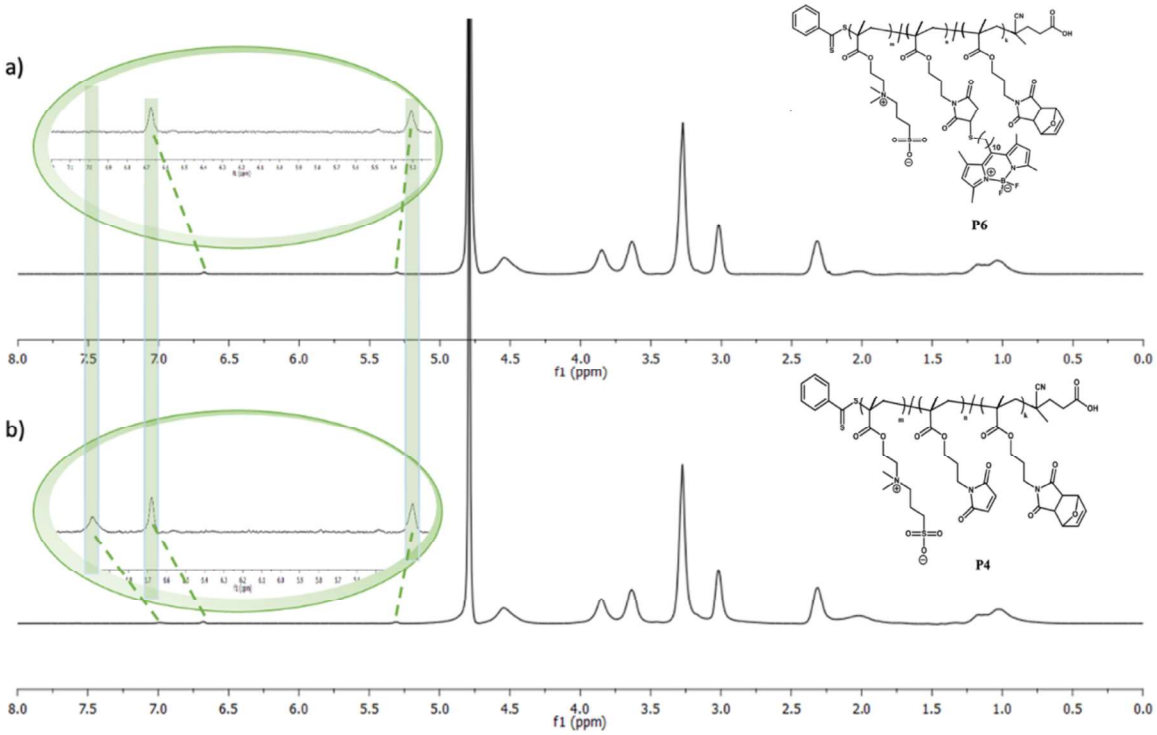

Figure 4.17. ^{1}H-NMR spectrum of (a) BODIPY-SH conjugated copolymer (**P6**) and (b) copolymer **P4** in D_2O.

The ratio of the SBMA monomer to non-activated moieties was 38:1 before conjugation, as evidenced by the integral ratio between the peaks at 4.45 ppm for the ester group of SBMA (designated as peak c) and 6.59 ppm for the distinctive proton signal of the cycloadduct (designated as peak a) (Figure 2.12). The copolymer P6 ratio remained 38:1 during the conjugation, which is proven by the same calculations (Figure 2.19).

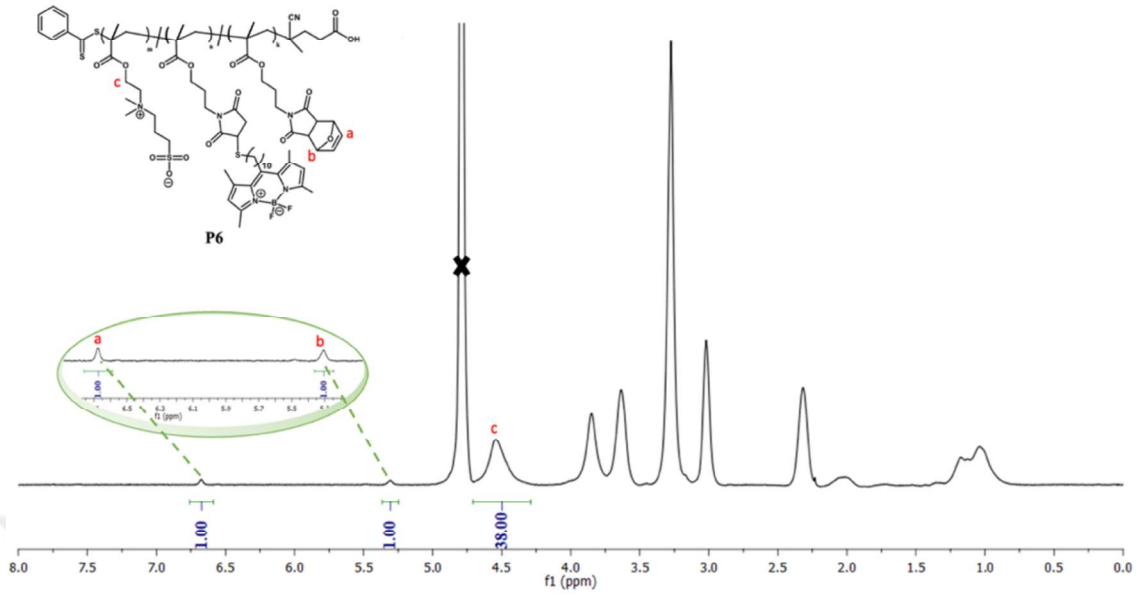

Figure 4.18. ^{1}H-NMR of BODIPY-SH conjugated copolymer (**P6**) in D_2O.

BODIPY-SH solubility was examined in a hexane-water mixture as evidence of the conjugation and water solubility of hydrophobic dye when coupled to the zwitterionic polymer. Hexane was used to dissolve free BODIPY-SH before combining it with water in a vial. Because hexane and water are immiscible, layer formation was observed inside the vial. The vial then objected to UV light, and the fluorescent green color was observed in the hexane layer, whereas the water layer was transparent as expected (Figure 2.20a). On the other hand, the dye became water-soluble when it was conjugated to a zwitterionic-based copolymer (P6), forming a fluorescent aqueous solution with a transparent hexane layer (Figure 2.20b).

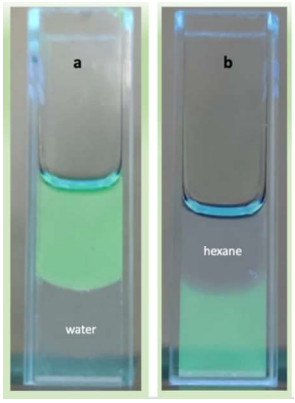

Figure 4.19. Photographs of UV irradiated vials (a) free BODIPY-SH insoluble in water, soluble in hexane, (b) BODIPY-SH becomes water-soluble when conjugated to hydrophilic copolymer **P4.**

UV-Vis and fluorescence spectra were used to analyze the physicochemical characteristics of the copolymer P6, as shown in Figures 2.21 and 2.22. As expected, the highest absorbance of the BODIPY-SH conjugated copolymer was at 492 nm. Moreover, a distinctive emission peak with a maximum of about 506 nm was visible in the fluorescence spectra, indicating the successful attachment of BODIPY-SH to the zwitterionic water-soluble polymer.

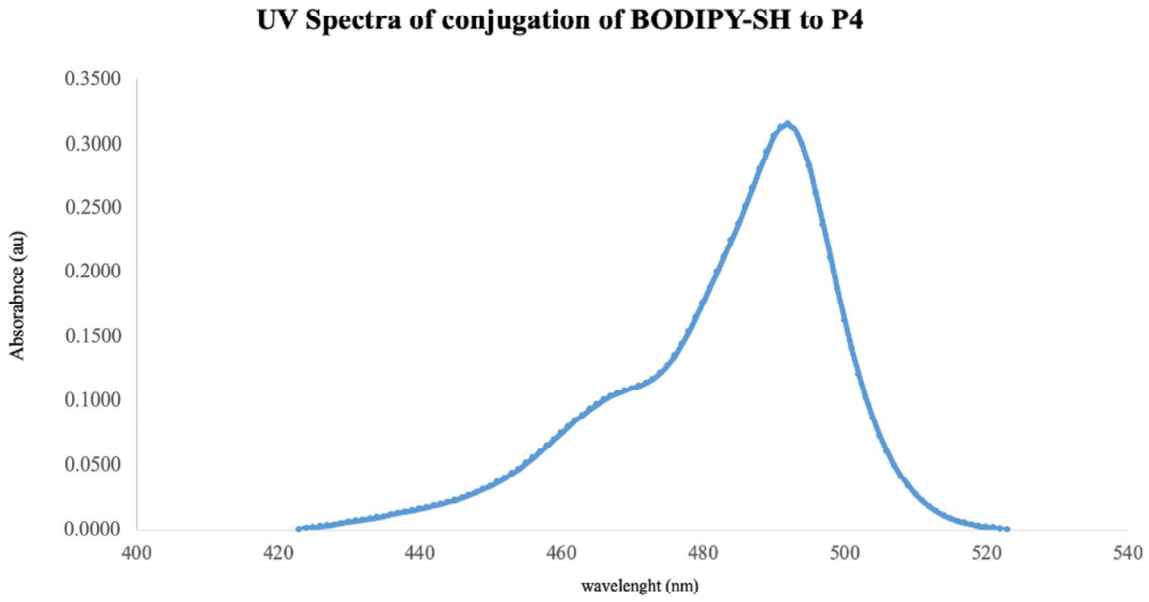

Figure 4.20. UV spectrum of **P6**.

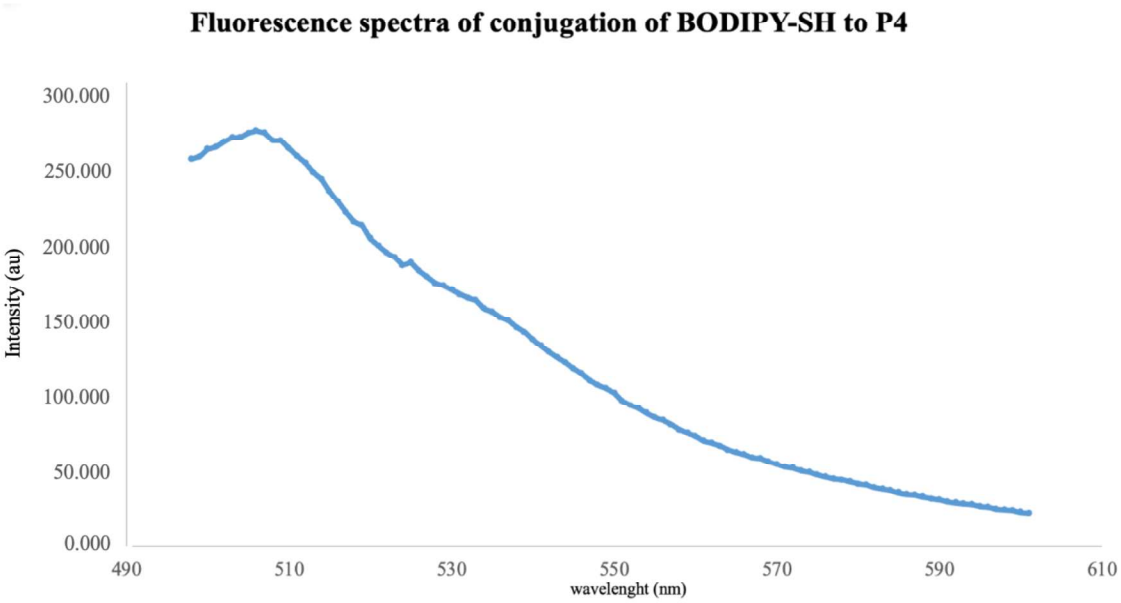

Figure 4.21. Fluorescence spectrum of **P6**.

5. CONCLUSION

In this project, a furan-maleimide-based cycloadduct was used to synthesize a methacrylate monomer with a protected maleimide unit. Using the reversible addition-fragmentation chain transfer (RAFT) polymerization, this monomer was copolymerized with the commercially available zwitterionic monomer, SBMA. Three different copolymers were successfully synthesized with different monomer equivalence ratios. A 5% latent-reactive polymer-containing polymer was used for further modifications. By heating the polymer, 50% of the maleimide groups were obtained quantitatively in their original form. The maleimide groups were conjugated with a thiol-containing hydrophobic fluorescent dye called BODIPY-SH by 'click' chemistry to demonstrate that the designed polymer can be functionalized efficiently at room temperature and that the methodology is applicable for fabrication of drug delivery systems through chemical conjugation of a thiol-containing drug. Successful immobilization of the hydrophobic dye was proved by using 1H NMR spectroscopy and by the images showing the dye's solubility in an aqueous medium when attached to the zwitterionic polymer. Further modification can be done by using the double bond of 50% masked-maleimide moieties, which were not activated during the retro Diels-Alder reaction, on the polymer with a thiol-containing targeting group molecule using UV mediated thiol-ene click chemistry.

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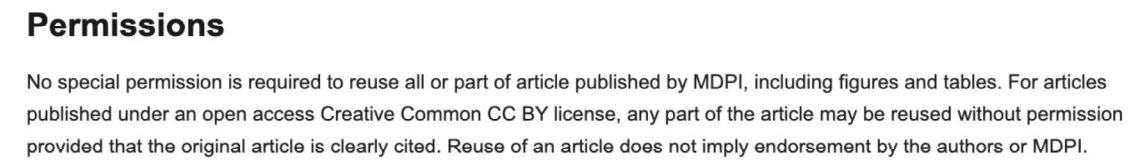

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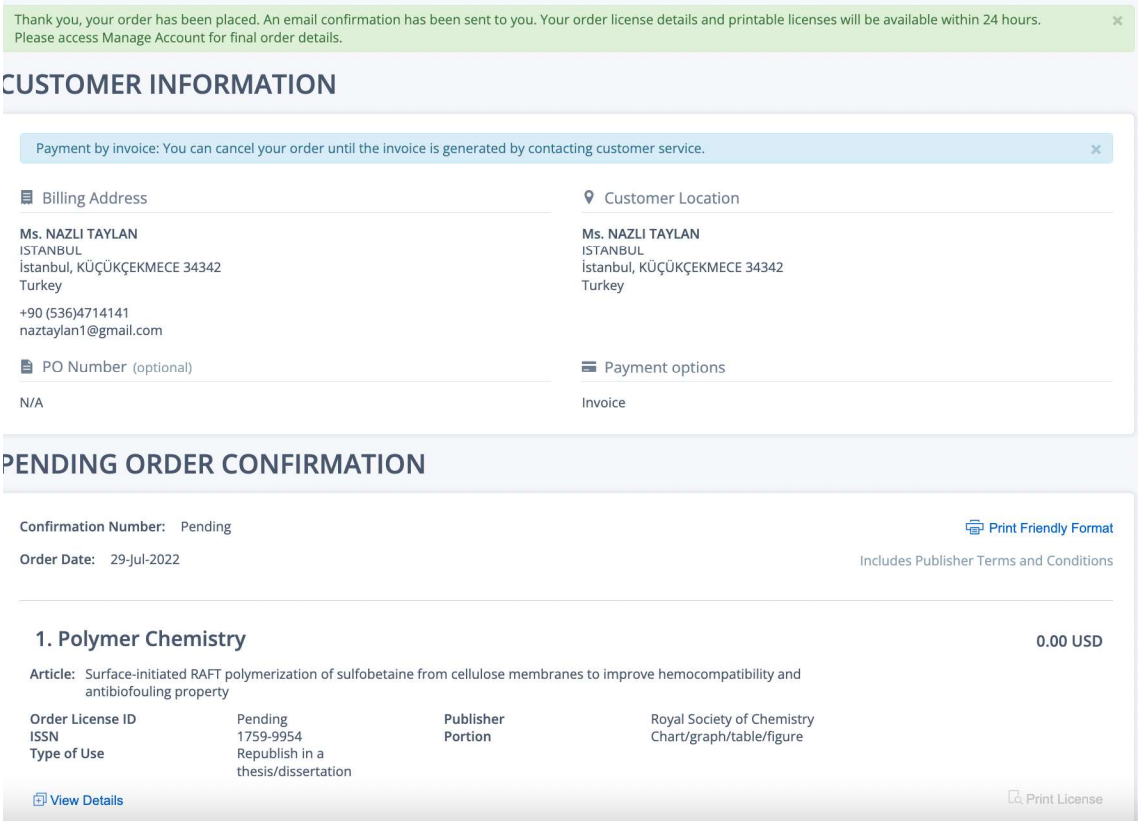

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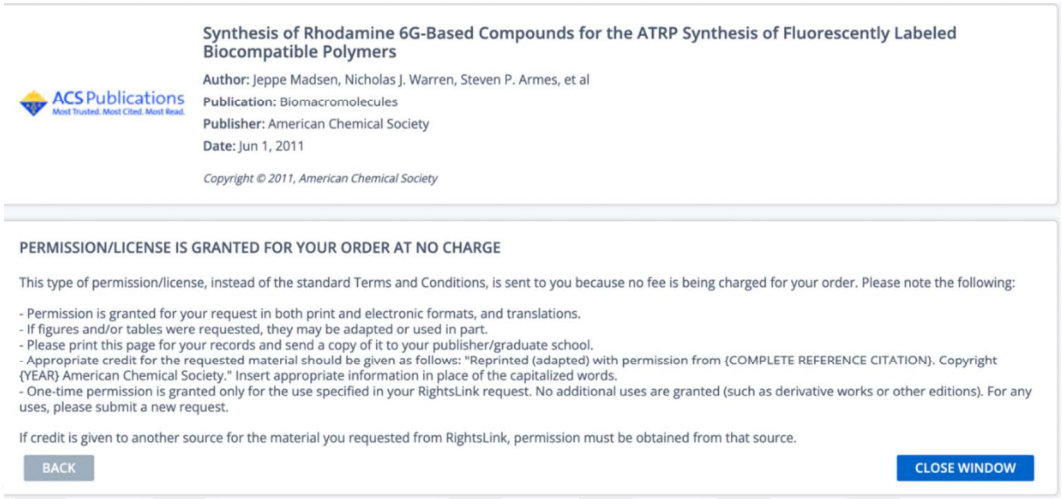

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Publication: Biomacromolecules
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Date: Jun 1, 2011

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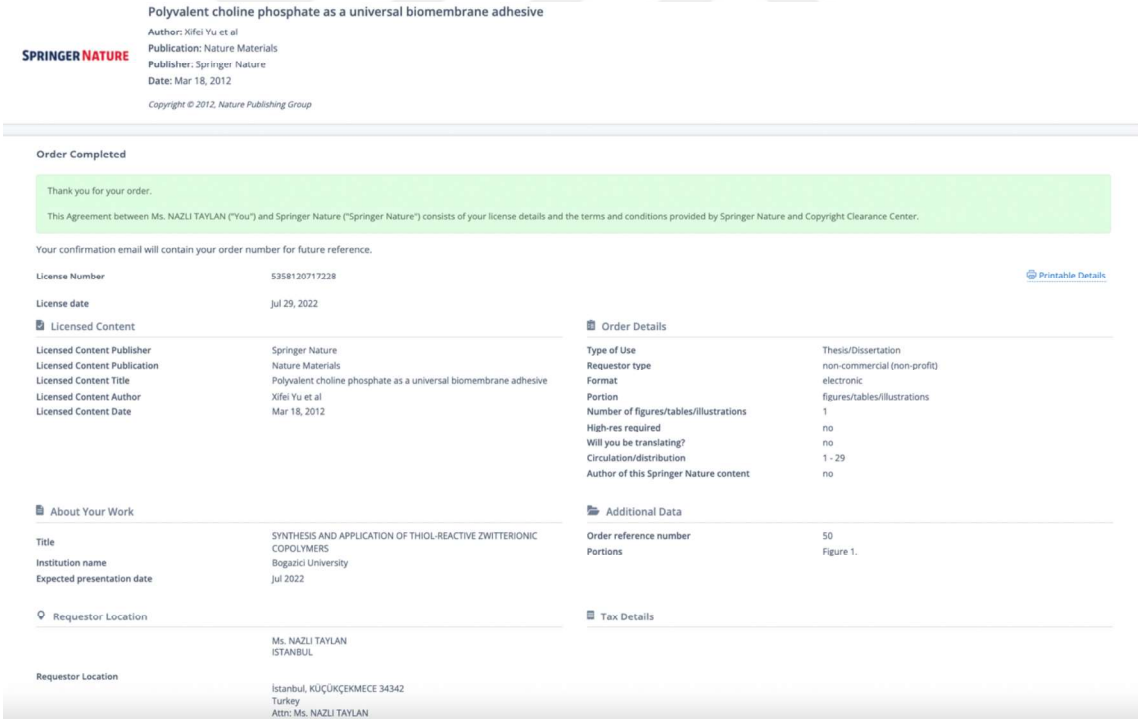

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Publication: Nature Materials
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Stefanie C. Lange,[†] Esther van Andel,[†,‡] Maarten M. J. Smulders,[†] and Han Zuilhof[*,†,§]

[†]Laboratory of Organic Chemistry, Wageningen University, Stippeneng 4, 6708 WE Wageningen, The Netherlands
[‡]Cell Biology and Immunology Group, Wageningen University, 6709 PG Wageningen, The Netherlands
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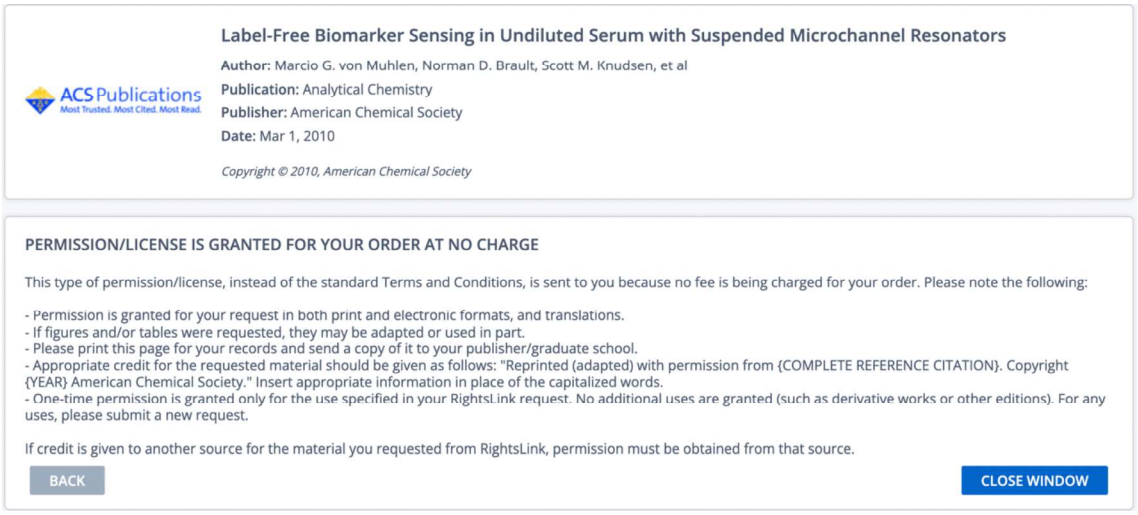

Label-Free Biomarker Sensing in Undiluted Serum with Suspended Microchannel Resonators

Author: Marcio G. von Muhlen, Norman D. Brault, Scott M. Knudsen, et al
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Date: Mar 1, 2010

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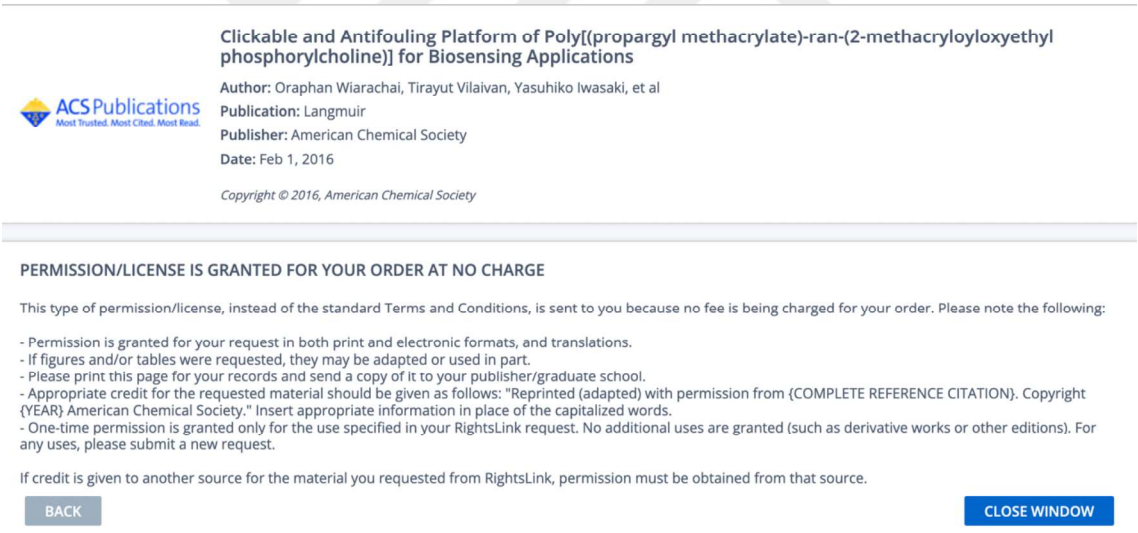

Clickable and Antifouling Platform of Poly[(propargyl methacrylate)-ran-(2-methacryloyloxyethyl phosphorylcholine)] for Biosensing Applications

Author: Oraphan Wiarachai, Tirayut Vilaivan, Yasuhiko Iwasaki, et al
Publication: Langmuir
Publisher: American Chemical Society
Date: Feb 1, 2016

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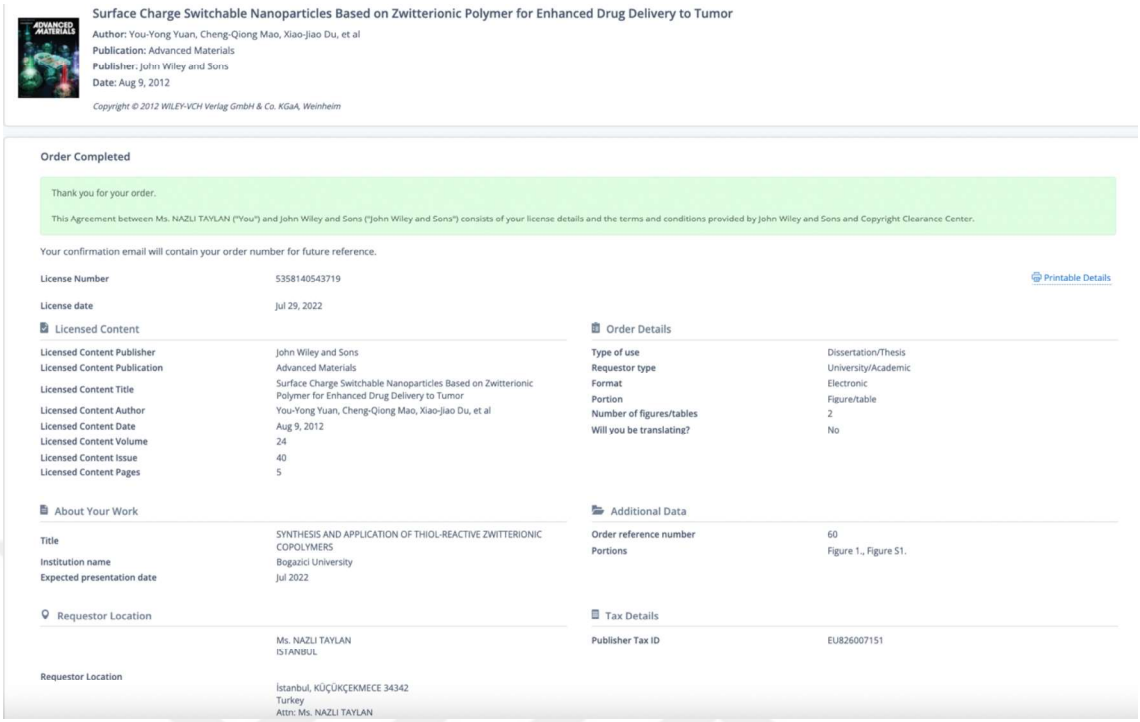

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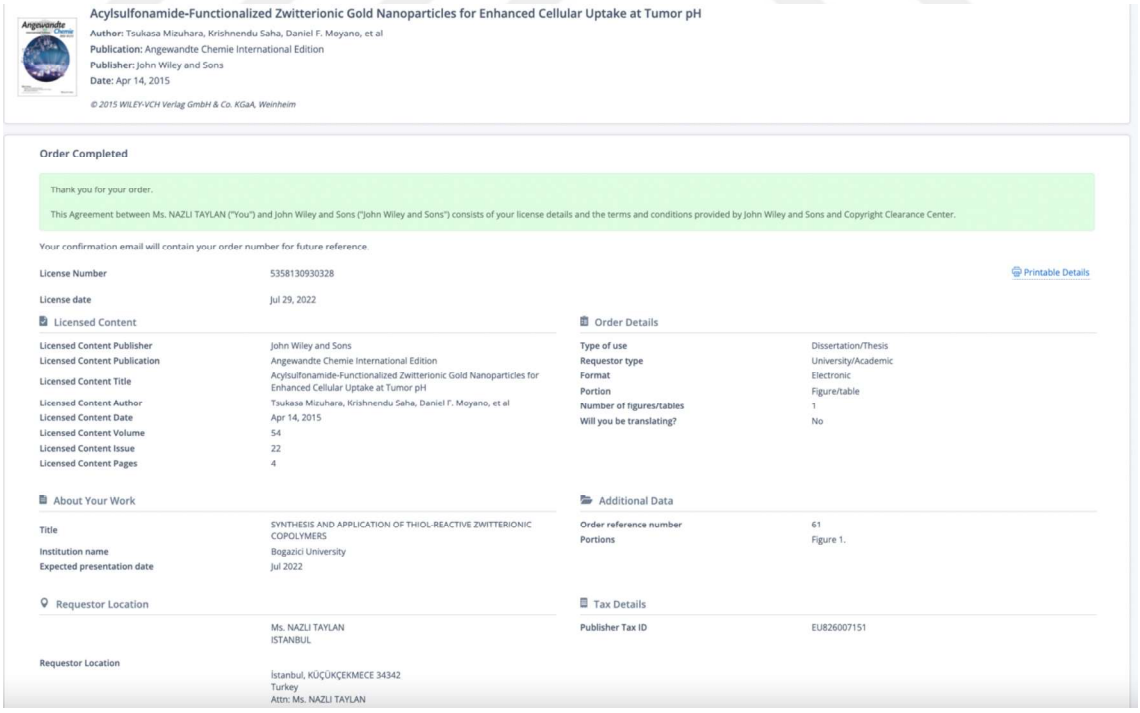

Acylsulfonamide-Functionalized Zwitterionic Gold Nanoparticles for Enhanced Cellular Uptake at Tumor pH

Author: Tsukasa Mizuhara, Krishnendu Saha, Daniel F. Moyano, et al
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Publisher: John Wiley and Sons
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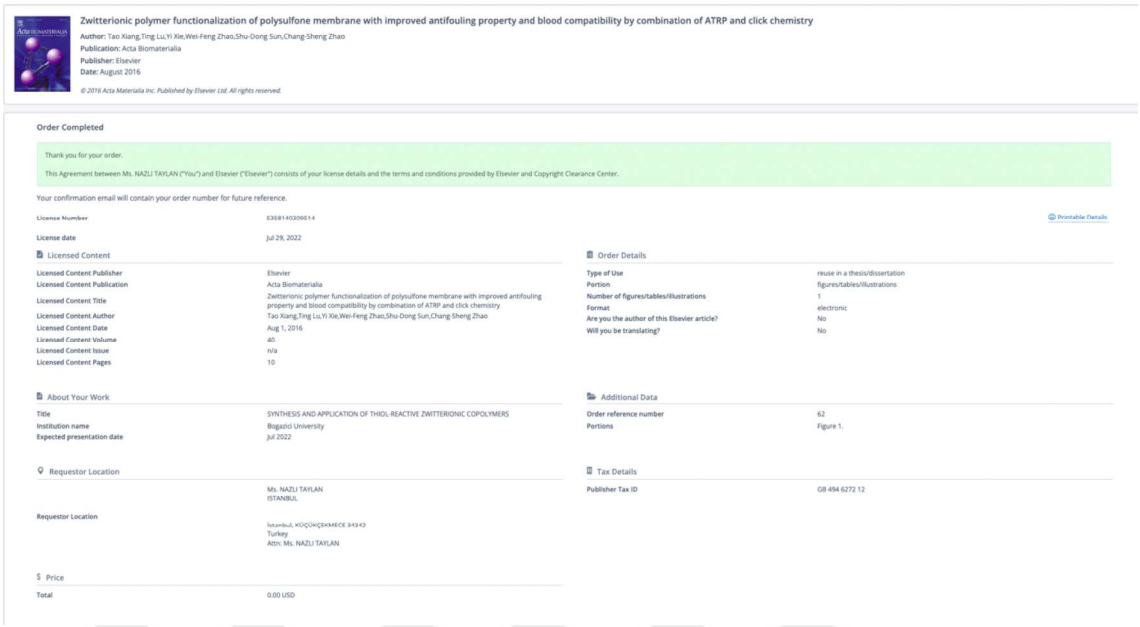

Zwitterionic polymer functionalization of polysulfone membrane with improved antifouling property and blood compatibility by combination of ATRP and click chemistry

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Publication: Acta Biomaterialia
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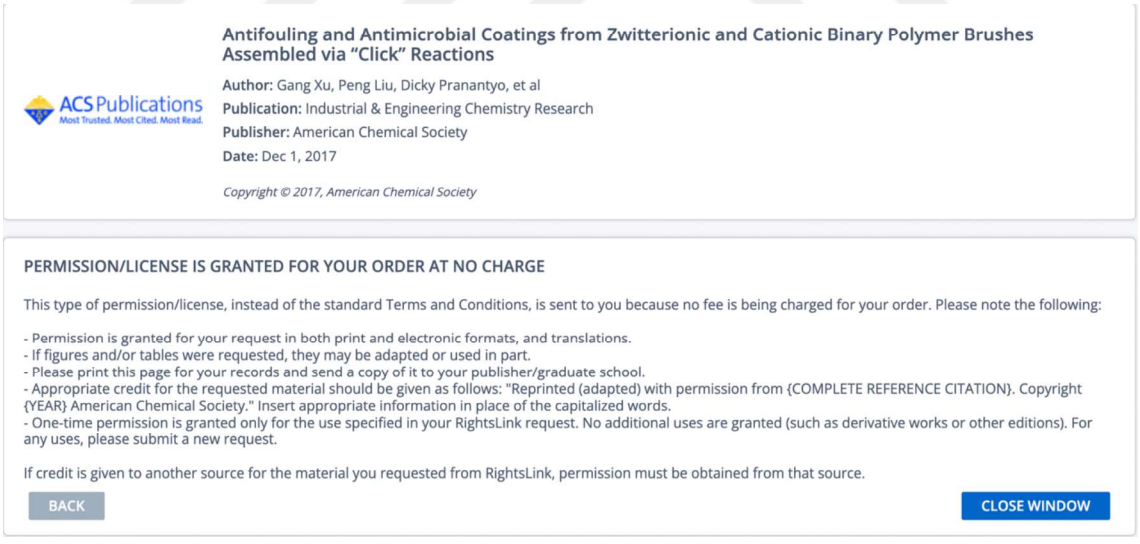

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Publication: Industrial & Engineering Chemistry Research
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Biomedical Hydrogels Fabricated Using Diels–Alder Crosslinking

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Publication: European Journal of Organic Chemistry
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Publication: Bioconjugate Chemistry
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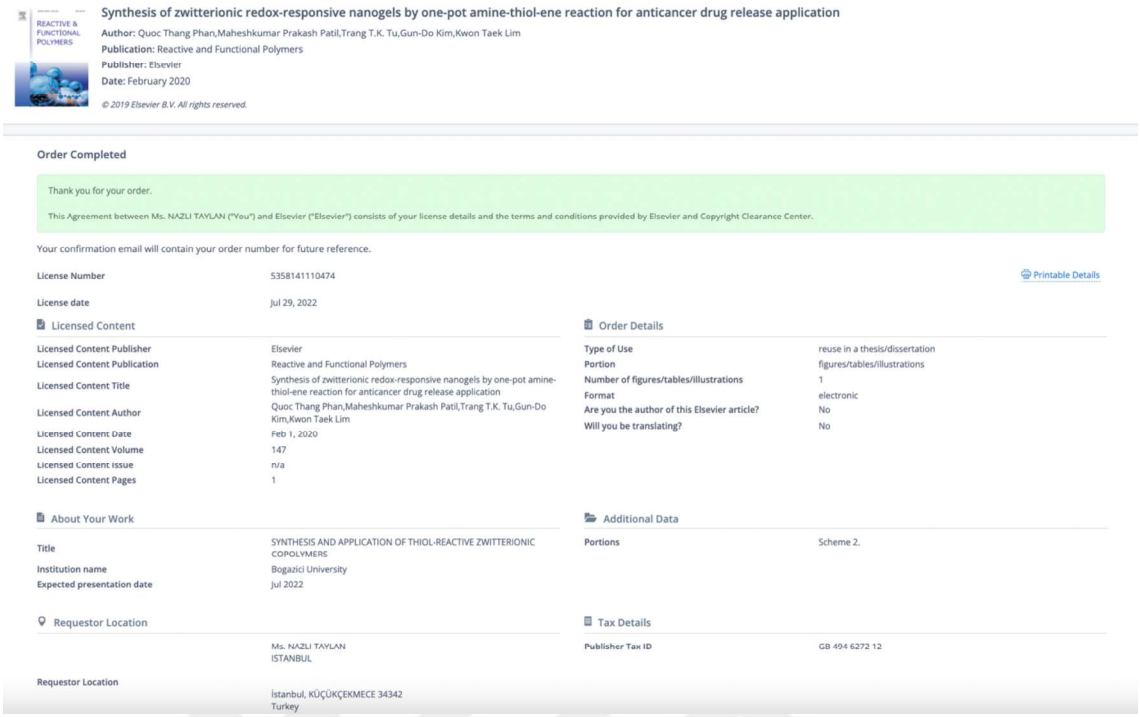

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Publication: Reactive and Functional Polymers
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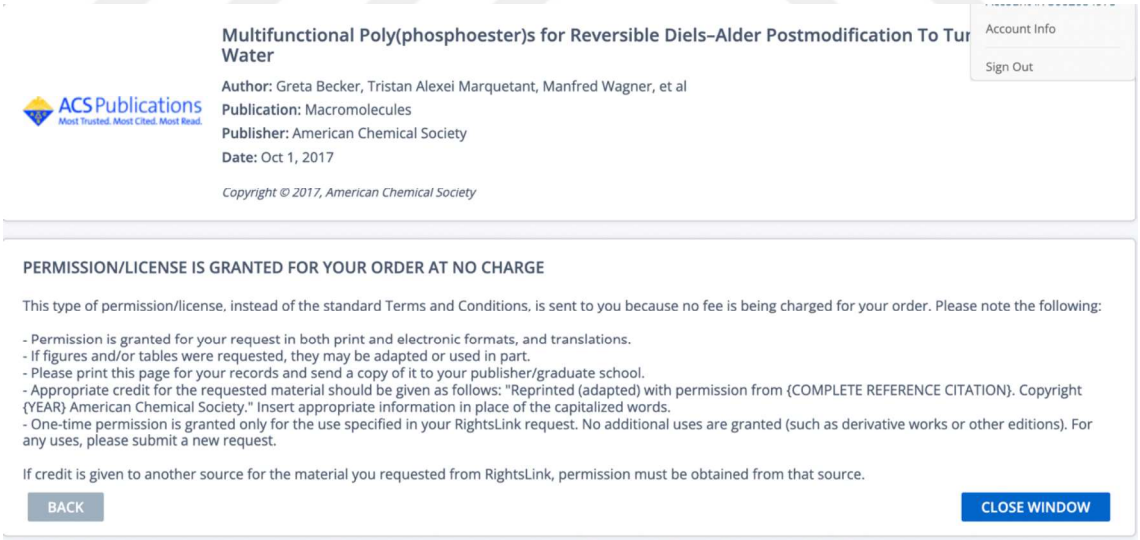

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Publication: Macromolecules
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