DRUG DELIVERY PLATFORM FOR TARGETING OSTEOSARCOMA

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Dedicated to my mother…

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ABSTRACT

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Drug delivery systems remain a challenge in the management of cancer and wide variety of diseases. Polymers containing different functional groups are widely used in the delivery of drugs. The molecular weight of the polymer is very crucial to achieve the targeted drug delivery and enhanced permeability and retention (EPR) effect. In this thesis, we utilized reversible addition-fragmentation chain transfer (RAFT) polymerization method to prepare high-molecular weight and water-soluble polymers which bear bisphosphonate groups along with anti-cancer agents on their side chains. Bisphosphonic acids have high affinity to the hydroxyapatite in the bone tissue, rendering them suitable ligands for bone targeting. The final constructs carrying chemotherapy agent linked to the polymer by activated carbonate group and targeting moiety will be evaluated *in vitro* for drug release and in terms of targeting efficiency.

ÖZET

OSTEOSARKOM İÇİN İLAÇ TAŞIYICI PLATFORM

İlaç taşıyıcı sistemler kanser ve bir çok çeşit hastalığın tedavisinde meydan okuyucu rol oynamaktadırlar. Farklı fonksiyonel grup taşıyan polimerler ilaç taşıyıcı sistemlerinde oldukça yaygın olarak kullanılırlar. Hedeflendirilmiş ilaç taşıyıcı sistemlerinin verimliliği ve geçirgenlik ve alıkonma yüksekliği (EPR) için kullanılan polimerlerin molekül ağırlıkları oldukça önemlidir. Bu tezde tersinir katılma-bölünme zincir transfer polimerizasyonundan (RAFT) faydalanarak yan zincirlerinde bisfosfonat grupları ve antikanser ajanları olan yüksek molekük ağırlıklı ve suda çözünebilir polimerler hazırlanmıştır. Bisfosfonik asitlerin hidroksiapetit ile olan yüksek afinitesi bu grupları kemik için uygun hedefleyici haline getirmiştir. Laboratuvar ortamındakı ilaç salımı, hedefleme verimi deneyleri, hedefleyici kısım ve aktif edilmiş karbonat ile bağlı olan kemoterapi ajanı bulunan son polimerik yapı üzerinde değerlendirilmiştir.

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AIBN	2,2′-Azobis(2-methylpropionitrile)
ALN	Alendronate
$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Dichloromethane
CTA	4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid
D_2O	Deuterated water
DMF	Dimethyl formamide
DMPA	2,2-Dimethoxy-2-phenylacetophenone
DOX	Doxorubicin hydrochloride
EPR	Enhanced Permeability and Retention
Et_2O	Diethyl ether
EtOAc	Ethyl Acetate
FT-IR	Fourier Transform Infrared
GPC	Gel Permeation Chromotography
MeOH	Methanol
MHz	Mega hertz
NEt_3	Triethylamine
NMR	Nuclear Magnetic Resonance
NHSMA	N-hydroxysuccinimide methacrylate
NHS	N-hydroxysuccinimide
PEG	Poly(ethylene glycol)
PEGMEMA	Poly(ethylene glycol) methyl ether methacrylate
RAFT	Reversible-addition Fragmentation Chain Transfer Polymerization
RT	Room Temperature
SEC	Size-Exclusion Chromatography
SCEMA	2-(N-succinimidylcarboxyoxy)ethyl methacrylate
TEA	Triethylamine
THF	Tetrahydrofuran

TLC	Thin Layer Chromatography
UV	Ultraviolet

1. INTRODUCTION

1.1. Cancer and Chemotherapy

Cancer is a leading cause of death in the world. According to World Health Organization statistics, 7 million people are died from cancer every passing year. Accordingly, it accounts for about 12.5% of deaths worldwide[1].

In a healthy body, cells grow and divide in a controlled way, extremely orderly fashion to replace those that have grown old or have been damaged and die by a process called apoptosis. Cancer occurs when there is a problem in these natural process and a group of the cells have lost ability to control growth through mutation. Cells divide rapidly to form tumors, and sometimes cancer cells spread to other part of the body through blood and lymph system.

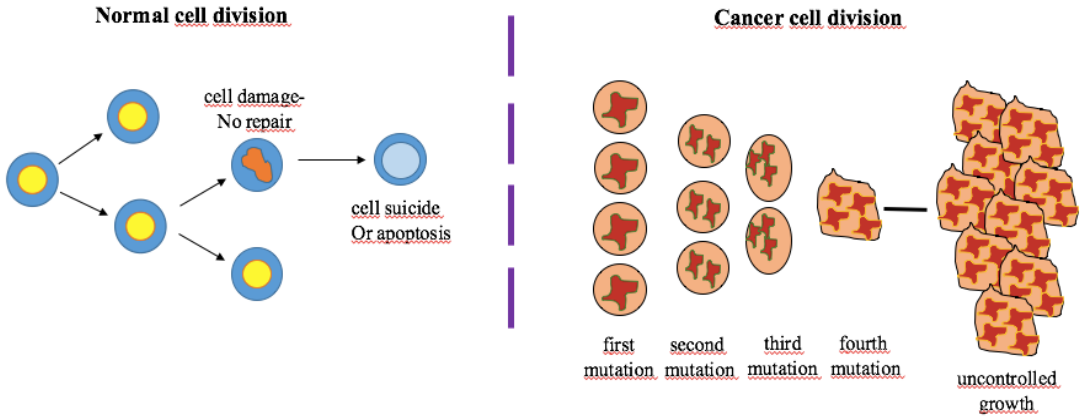


Figure 1.1. Cancer cell division.

Many different types of treatment methods for cancer exist. Treatments are used depend on the type, level of the cancer, location and patient's health. Some of the most common types of cancer treatments are surgery, chemotherapy, radiation therapy, hormonal therapy and targeted therapy. Chemotherapy, treatment of cancer using cytotoxic anticancer

agent remains the mainstay of therapy for malignant disease [2]. The main aim of the ideal cancer chemotherapy is to deliver the accurate amount of drug with desired controlled rate and for long duration of time to the tumor cells, while inhibit the normal cells to catch the desired therapeutic response [1]. Yet, traditional chemotherapy is limited in terms of a narrow therapeutic index, significant toxicities and frequently acquired resistance. In addition to these inadequacies, there are some significant drawbacks of chemotherapy which are seen in patients, such as vomiting, loss of appetite, constipation and diarrhea. Fever, fatigue and hair loss are other common side effects experienced by chemotherapy patients [3].

In order to overcome these drawbacks, drug delivery systems should hold adequate amount of drug by rooting out the drawbacks like drug resistance, altered biodistribution, biotransformation as well as clearance of chemotherapeutic drugs from the body.

1.2. Drug Delivery Systems

Drug delivery system is a challenge in the management of cancer. Drug delivery strategies for cancer treatments diversify based on the type and site of the cancer [4]. Conventional chemotherapy with small molecule chemotherapeutic drugs has been used for lots of types of cancer for a very long time. However, the therapeutic efficacy remains less successful, due to the fact that conventional chemotherapy has poor tumor selectivity, rapid clearance, toxicity and significant adverse side effects which hampered the use of high drug doses. The enhancement of tumor-targeted chemotherapy with macromolecules is very crucial in terms of more successful treatment. The most important breakthrough which leads to develop DDSs is revealing unique anatomical structure of tumor sites called as enhanced permeability and retention (EPR) effect. Consequently, DDSs is developed based on this unique phenomenon and some of them are illustrated in figure 1.3.

The enhanced permeability and retention (EPR) effect is a unique phenomenon of solid tumors. According to this phenomenon, there are some important anatomical and pathophysiological differences between normal and tumor tissues. Tumor tissues have higher vascular density, larger gap between endothelial cells and lack of effective lymphatic drainage compared to normal tissues [5]. Accordingly, macromolecular drugs can

accumulate and retain in solid tumor tissues selectively without spreading to normal tissue. EPR based chemotherapy method is thus becoming a breakthrough strategy to develop the delivery of anticancer agents to tumor site [6]. Understanding EPR effect well is very important in the progress of developing drug delivery systems.

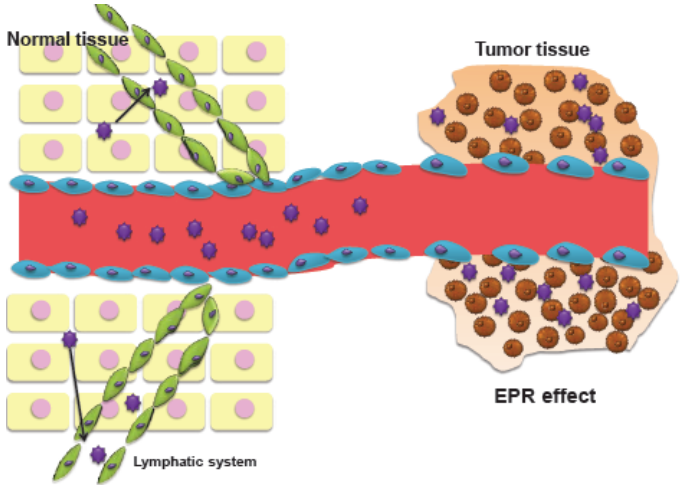


Figure 1.2. Diagrammatic representation of EPR effect. [6]

Due to the fact that conventional chemotherapy has many limitations in terms of therapeutic effect and patient's convenience and compliance, novel drug delivery systems have been developing to overcome drawbacks of traditional chemotherapy for decades [1]. In this concept, the major advantages of polymers are their great versatility in terms of structural view, the opportunity to gather hydrophobic and hydrophilic components. Additionally, by the means of the polymer-polymer, polymer-drug, polymer-solvent interaction, there are lots of different ways to prepare systems with desired properties and functions. Moreover, DDSs provide opportunity to release and deliver of more than one chemotherapeutic drugs to the site of action, and also they improve the bioavailability of low soluble drugs. These systems can be classified according to the function of structure and the release mechanism such as membrane based systems, matrix based systems, hydrophilic matrices, stimuli responsive systems and polymer-drug conjugates and their releases are controlled by latter mechanism respectively, drug diffusion or osmotic pressure, drug diffusion or matrix erosion, matrix swelling or matrix slow dissolution, changes in stimuli such as temperature or pH and, chemically [7,8].

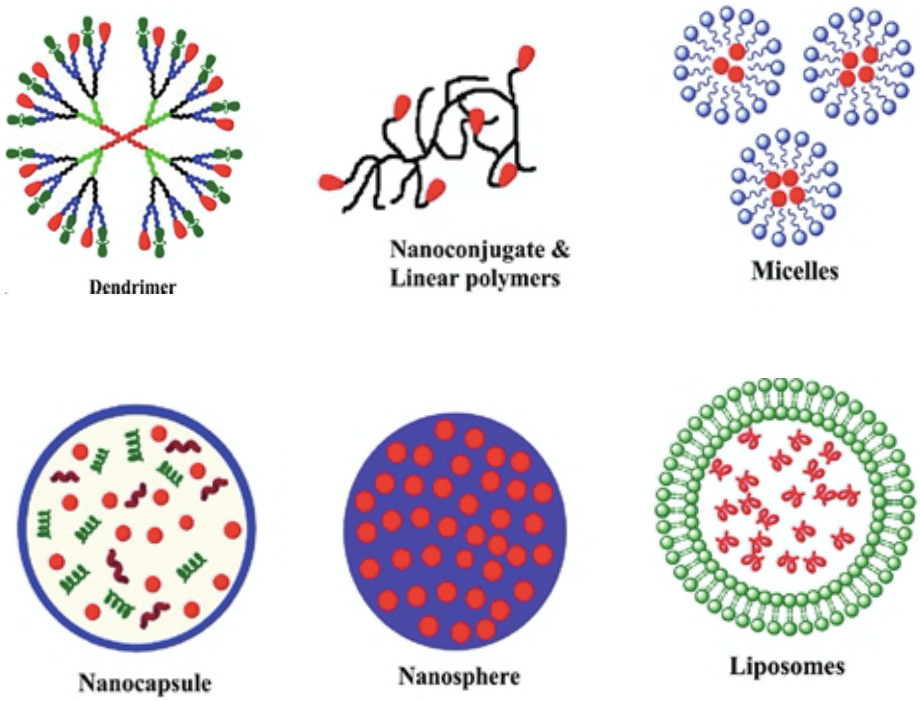


Figure 1.3. A schematic representation of some drug delivery system platforms [9].

1.2.1. Linear Polymers as Polymer-Drug Conjugates

Covalently bond polymer-drug conjugates are a specific type of DDS for binding drug or chemically active compound such as peptides, enzymes and hormones due to the fact that covalently linked drug on the macromolecular backbone can release according to the physiological condition [10]. The idea of covalently bond polymer-drug conjugates was revealed by Helmut Ringsdorf in 1975. This significant model is based on the covalent bond between the drug and polymer backbone by the means of labile bond. According to the idea of this model the biodegradable or bio-stable polymer backbone has three different groups; and each of them have special mission for effective drug delivery aim. Hydrophilic unit which makes pharmacologically active polymer soluble and non-toxic, second part in which the drug linked to the polymer backbone, and the third region is a targeting group so that the polymer conjugated molecule goes through the site of action [8]. Hence, one of the main functions of using polymeric carrier is to transport drugs to the site of action without being exposed to transformation by the body whereas contain a bond which only breaks under

physiological conditions. Therefore, these polymeric structures have been trying to improve pharmaceutical profile and the stability of a drug, ensure its sufficient concentration, facilitate the accumulation of a drug at a specific site, enhance maximum biocompatibility, minimize side effects, and increase the exposure time in the target cell [11]. This conjugation is not only for drug delivery applications but also very important for such applications like tissue engineering, biosensors and cell cultures.

A lot of polymer-drug conjugates have been synthesized by the means of utilizing water-soluble linear polymers. The most frequently used polymer-drug conjugates generally are based on poly(ethylene glycol) (PEG) and N-(2-hydroxypropyl)methacrylate (HPMA) copolymers which lead widespread use and versatility. PEG-Drug conjugates' PEG parts have lots of advantages, such as biocompatibility and commercially availability. In addition, these PEG polymers can be synthesized with narrow molecular weight and molecular weight distribution. The most common conjugations of PEG are performed by coupling to the end chains. For example, N-hydroxysuccinimide (NHS) esters or aldehydes ensure conjugation to the amine of lysine residues while maleimides react readily with the thiol cysteine residues. In addition, some functionalities on the PEG lead conjugation to biomolecules such as, proteins and antibodies. A number of other conjugation chemistries have also been applied on the PEG. These significant properties make PEG-drug conjugates attractive in the fields of pharmaceutical applications [12].

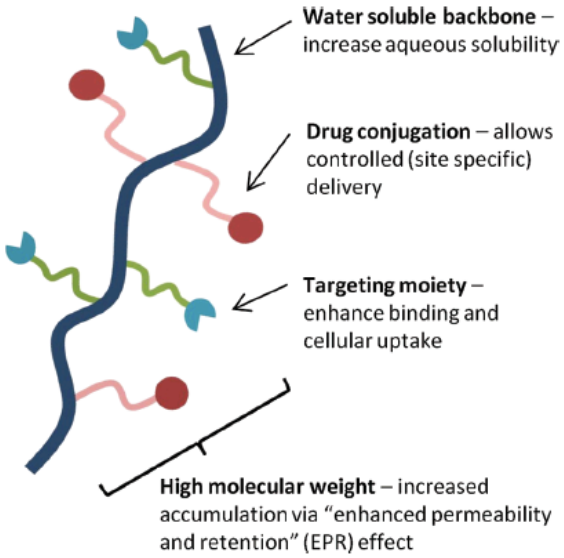


Figure 1.4. General scheme of a drug delivery system via polymer-drug conjugates [12].

1.3. Reversible Addition Fragmentation Chain Transfer (RAFT) Polymerization

Controlled free radical polymerization (CRP) techniques facilitate the synthesis of macromolecules with a level of control approaching which of the more traditional living techniques, such as anionic and group transfer polymerization, whereas possessing the versatility and durableness of conventional free radical polymerization.

RAFT operates on the principle of degenerative chain transfer and fundamentally has such differences from both stable free radical polymerization (SFRP) and atom transfer radical polymerization (ATRP) [13]. Successful RAFT is based on appropriate choice of a so-called RAFT agent or RAFT chain transfer agent (CTA) consisting of thiocarbonylthio group with substituent “Z” and “R” groups. More generally, the generic form of chain transfer agent is shown in Figure 1.5.

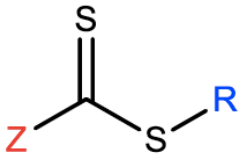


Figure 1.5. General molecular structure of RAFT agent, bearing thiocarbonylthio group with “Z” and “R” substituents [14].

The mechanism of RAFT process allows an equilibrium in which propagating polymer chains grow evenly with respect to monomer conversion. The proposed mechanism of RAFT polymerization is shown in Figure 1.6. In RAFT process, the activation and deactivation reactions form equilibria and are referred as chain-transfer reactions [15]. This method has significant advantages such as, broad functional group and solvent tolerance, compatibility for broad range of monomers and void of requiring any toxic metal catalysts [16]. The equilibrium between active propagating chains and passive thiocarbonylthio-bound chains ensures equal probability for all chains in order to grow, resulting in polymers with low polydispersity. RAFT polymerization takes advantage of the initiation and polymerization circumstances of the unmediated radical polymerization techniques. In order to achieve molecular weight and polydispersity control, it is necessary to add appropriate amount of RAFT agent to a polymerization mixture. In addition, R and Z groups

on the RAFT agent affect the equilibrium and quality of the molecular weight control in any given polymerization reaction [14].

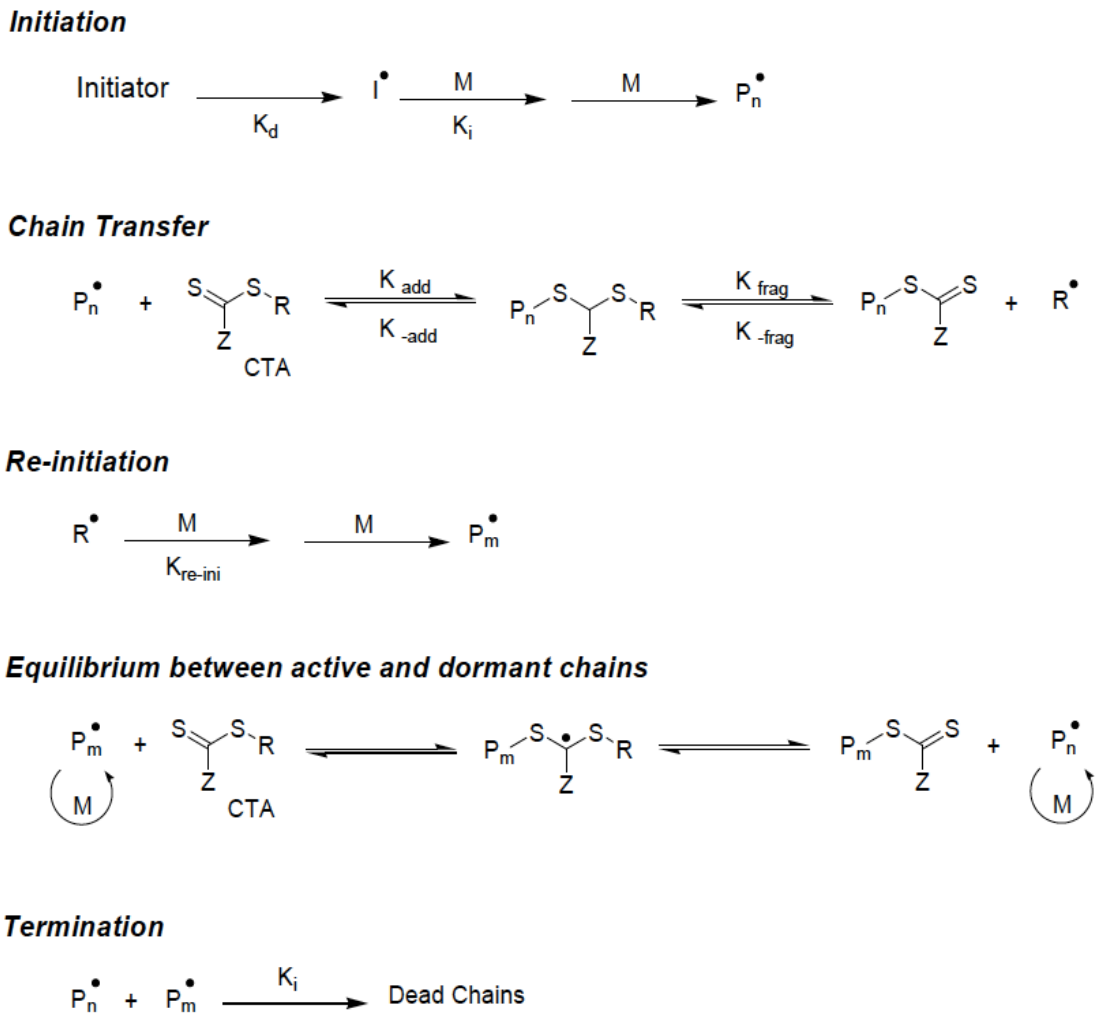


Figure 1.6. General mechanism of the RAFT polymerization [13].

1.4. Synthesis of Functional Polymers via Functional Group-containing Monomers

Rapidly demand of functional polymers in different interdisciplinary areas of research has highlighted the need for synthetic tools to develop advanced polymers bearing various functional groups [18]. Functional polymers are very effective molecules which

bear reactive functional groups on the structure. These reactive functional groups allow polymers to the attachment of different chemical compounds, such as drug and targeting moiety onto the structure backbone.

Synthesis of functional side chain polymers has attracted considerable attention due to the fact that these promising materials allow development of scaffolds enabling multivalent interaction. Therefore, these side chains can be considered as ligands which can bind certain groups because of these interactions [19,20]. In drug delivery systems, these side chains have a very important role for binding targeting moiety and drug onto the scaffold.

Polymers bearing reactive functional groups which react with amines have an important role in some applications such as bioconjugates or surface modifications [21]. N-hydroxysuccinimide esters and pentafluorophenol esters have very considerable role among other amine reactive group containing molecules [22].

1.4.1. Activated Esters

Ringsdorf and Ferruti revealed that the feasibility of activated ester chemistry for synthesis of reactive polymers. Later on, polymer scientists started to work on various activated ester based monomers which can be polymerized under mild conditions [23]. Active ester modified polymers are highly reactive when they are prone to reaction with amine containing biomolecules. Lots of different type of active ester bearing monomers and polymers have been synthesized via variety of different polymerization techniques. Due to the electron withdrawing groups, activated esters show great reactivity in the presence of amine bearing molecules. Some of the most common examples of active ester groups are illustrated in Figure 1.7. Activated esters are useful groups for covalent binding of functional amines based on a stable amide bond formation which is the basis of most of biological system [18].

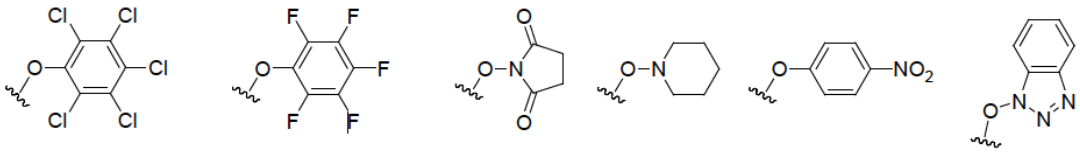


Figure 1.7. Examples of active ester group.

N-Hydroxysuccinimidyl (NHS) esters are one of the most widely studied one among the other active esters [24]. A lot of functional groups including N-hydroxysuccinimide-based activated esters which allow the introduction of amine bearing molecules that allow 'click' chemistries have been efficiently incorporated into polymers as pendant groups [25]. NHS-ester-based reactive polymers are very resistant to hydrolysis and also, they are subjected to nucleophilic aminolysis with primary and secondary amines under mild conditions to generate polyacrylamide derivatives [18]. In the fields of drug delivery applications, these features make NHS-based activated esters very favorable to attach targeting group.

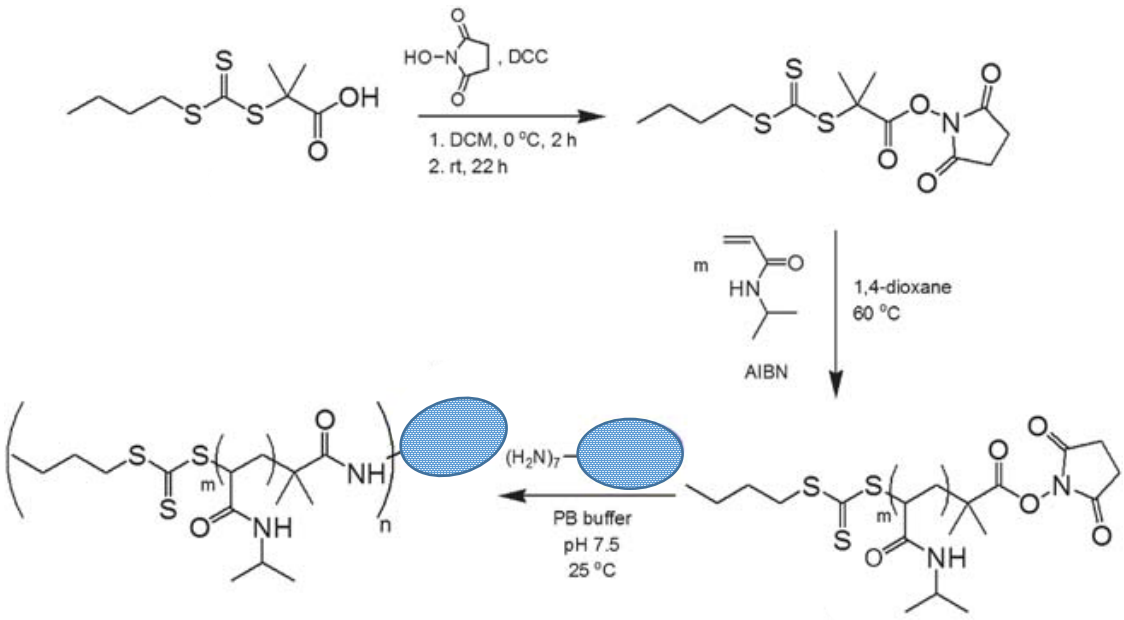


Figure 1.8. Synthesis of amine reactive end-group containing polymer [26].

NHS containing polymers can be synthesized via different polymerization techniques such as Atom Transfer Radical Polymerization (ATRP) and Reversible

Addition-Fragmentation Chain Transfer (RAFT). Sumerlin et al synthesized an amine reactive polymer by using activated ester group bearing RAFT agent. In order to synthesize thermoresponsive polymers for conjugation with amine bearing proteins they used RAFT polymerization of N-isopropylacrylamide with an active ester containing RAFT agent [26].

1.4.2. Carbonates

Molecules bearing carbonate functional group can covalently bind to amine group to form carbamate linkage by undergoing fragmentation. Due to the hydrolyzable feature of carbamate linker, it has been used in design of prodrugs but has not been widely used and investigated in polymer based molecules [27]. For this reason, there are few examples of carbonate containing amine reactive multifunctional polymeric materials in the literature. Lately S. Diamanti et al are investigated pendant carbonate moiety bearing functional polymers. In that study, they synthesized carbonate groups by using postpolymerization functionalization technique in order to prepare multifunctional polymer brushes (Figure1.7) [28].

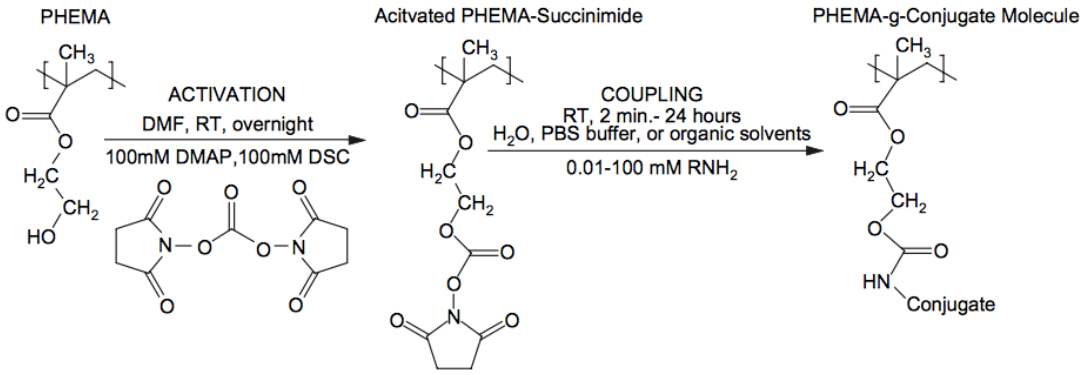


Figure 1.9. Functionalization of PHEMA side chain using DSC activation and subsequent amination [28].

In drug delivery applications, attachment of a drug to the backbone scaffold by using various functional group containing monomers is another important part besides attachment of targeting moiety. Carbonate containing functional monomers are one of the most suitable derivatives for binding of amine bearing drugs and targeting groups in order to form cleavable carbamate linker between drug or targeting moiety and the monomer. The stability

of the drug-polymers linkage is very crucial under different biological conditions. This linkage possesses the inherent biodegradability property to release the payload, although it is very stable in the plasma. Based on the stability and cleavage conditions, the carbamate linkage has highly attracted attention in polymer therapeutics. In addition, such conjugation linkers are self-cleaved by β -elimination reaction in a highly predictable manner [29,30]. Structurally, the carbamate functionality is amide-ester hybrid characteristics and generally they show considerable chemical and proteolytic endurances. Carbamates are mostly used as a peptide bond surrogate in the fields of medicinal chemistry, due to the fact that they have chemical stability and capability to permeate cell membranes [31].

1.5. Orthogonally Functionalizable Polymers

Chemoselective reactions are very important due to interference of the other functional groups' reactions in the medium. This feature is defined as orthogonal reactions. In biological nature chemical reactions take place in an orthogonal nature. For example, enzymes bind or react with substrate directly even if there are lots functional group in the medium. Hence, functional polymeric materials can imitate to biological systems thanks to the orthogonal functionalization.

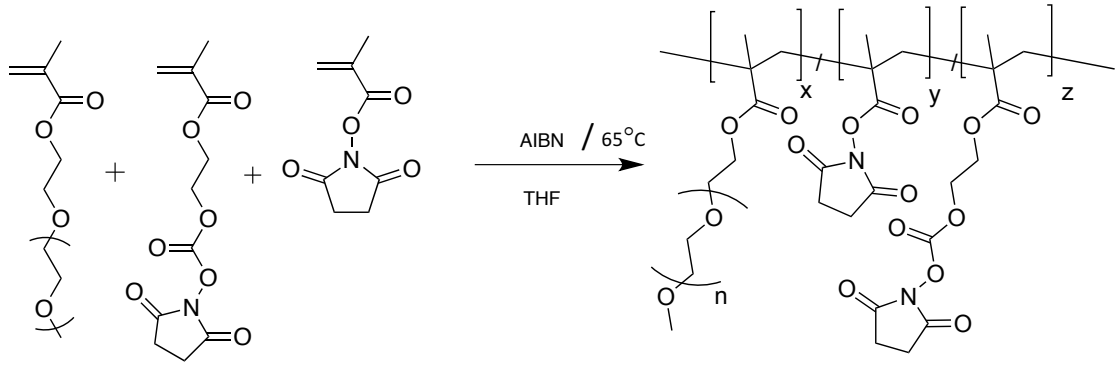


Figure 1.10. Copolymerization of SCEMA monomer with N-hydroxy succinimide-based monomer (NHSMA) [32].

In the fields of drug delivery systems, orthogonal functionalization of polymers is very important due to the fact that it provides an opportunity to attach targeting moiety and drugs onto the polymer backbone without interfering each other. The choice of the linkers to bind drug and the targeting group is crucial for such application because the targeting group should remain stable on the polymer backbone, therefore the linker should be nonmetabolizable, and the drug should be released as desired. For instance, Sanyal et al synthesized a novel N-Hydroxy succinimide-based carbonate monomer that allows direct synthesis of functional polymers on the side chain [32]. This novel carbonate monomer was copolymerized with poly(ethylene glycol) methylether methacrylate via free-radical polymerization in the presence of azide-containing monomer and N-hydroxy succinimide-containing activated ester monomer in order to prove reactivity and orthogonal functionalization of the novel carbonate monomer .

1.6. Bisphosphonates

The skeletal system of the human body consists of bone matrix, minerals and cells. If there are any defects in these constituents, it may hinder skeletal developments, that causes bone disorders [33,34]. One of the most promising treatments of bone diseases such as osteosarcoma is bisphosphonate treatments. Bisphosphonates are small compounds with two phosphonates and, homologue of pyrophosphate derivatives which lower calcium levels in the body [35]. They are very useful in preventing many diseases such as metabolic bone disorders, Paget's disease, osteoporosis and metastatic bone disease. In addition, they can be used as imaging agent [36].

Pyrophosphate is a great complexing derivative, and it exist in all living organisms. The unstable P-O-P group of pyrophosphate has an affinity towards hydroxyapatite which is one of the mineral existing in the bone. Many literature examples are demonstrated that pyrophosphate (PP) is capable of hindering calcification by binding to hydroxyapatite crystals. However, due to the unstable feature of P-O-P linkage, it fails to prevent dissolution of biological apatite sufficiently [35]. It is very important to think about all the possible interactions of a drug until it comes to the main treatment site. The major drawback of pyrophosphate is that when it is administered orally, it cannot be an active due to the hydrolysis in gastrointestinal tract. Yet, bisphosphonates are chemically stable derivatives

of inorganic pyrophosphates in which two phosphate groups are bind by esterification. Thus, bisphosphonates have the same chemical activity with pyrophosphates but their stability greater than these molecules [37,38,39].

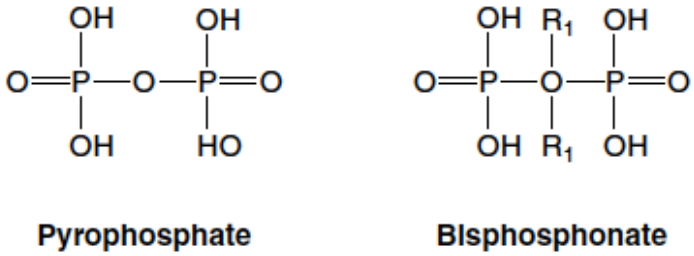


Figure 1.11. Chemical structures of pyrophosphate and bisphosphonate.

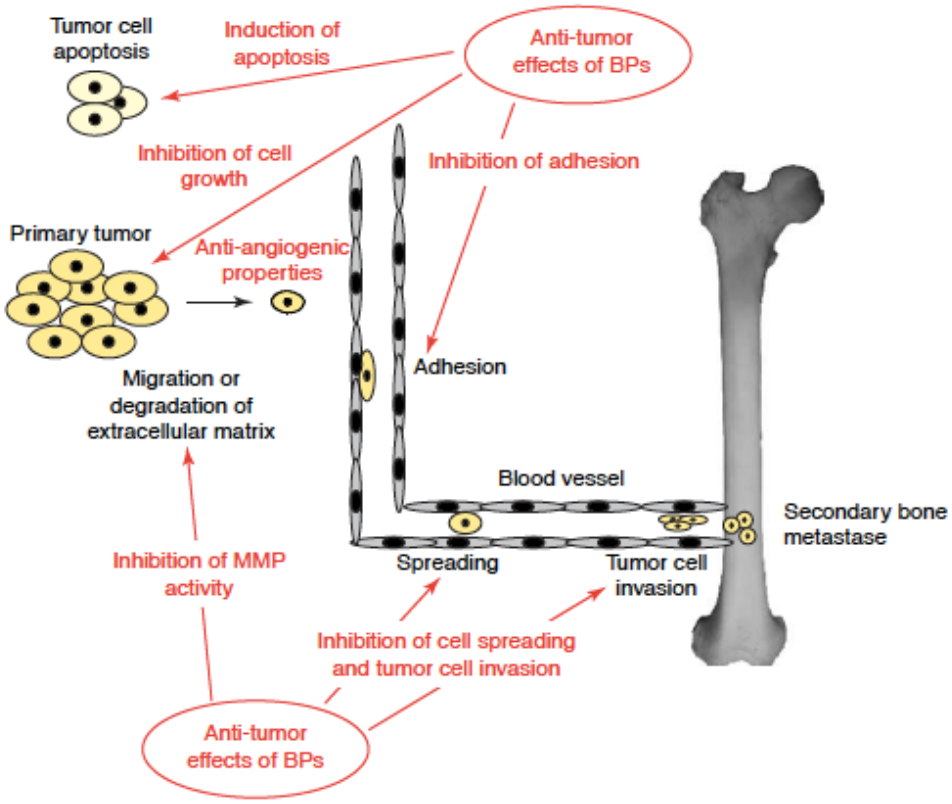


Figure 1.12. Bisphosphonates: Mechanism of action [40].

There are two phosphonates in germinal carbon of bisphosphonates which result in having affinity to HA. The gap between the two deprotonated hydroxyls of the two phosphonates (2.9-3.1 Å) is analogues the distance between two calcium-chelating oxygen atoms in HA. Hence, bisphosphonates are ideal molecules for bidentate binding to Ca^{2+} in

HA. Additionally, the presence of -OH or -NH_2 groups on the R_1 shows improved affinity to HA and greater anti-resorptive properties. Thus, all of these features provide to having an affinity to HA mineral in the bone [35].

Bisphosphonates can prevent tumor proliferation by the means of several mechanism: the induction of cell apoptosis [41], the preventing cell growth [42], the induction of cell adhesion and invasion [43] (Figure 1.10.).

1.6.1. Bisphosphonates for the Treatment of Bone Cancer

Many types of cancers tend to spread to the other parts of the body, one of the most common sites of metastasis is bone arising from the breast or prostate cancer generally resulting skeletal complications such as bone pain, impaired mobility, spinal cord compression and hypercalcaemia [44]. It is concluded that there are 1.5 million cancer patients having bone metastasis. For bone pain therapy, it is proved that bisphosphonates are very effective as supplementary for radiotherapy. Due to the fact that bisphosphonates bind avidly to HA bone mineral surfaces and are selectively diffused by osteoclast where they slow their activity, they are very effective targeting candidate for bone cancer [45]. On the other hand, bisphosphonates are not retained in the bone, contrarily they are cleared from the blood circulation by the means of effective renal excretion. Apart from the ability to prevent calcification, bisphosphonates inhibit and slow hydroxyapatite breakdown, hence effectively prevent bone resorption [46]. This special property of bisphosphonates makes them considerable clinical agents.

In the recent literature examples, the bisphosphonates also have a function to limit both osteoblast and osteocyte apoptosis [47,48]. On the other hand, there are some evidences that are revealed by preclinical and clinical studies regarding to bisphosphonates also have antitumor activity which makes significant contribution to their therapeutic efficacy. These antitumor effect vary from molecules to molecules according to the groups on it. The N atom bearing bisphosphonates have been clinically outstanding to the first generation equals [40]. According to some cell culture experiments bisphosphonates conduce to apoptosis in different human tumor cell lines such as breast, prostate, lung, osteosarcoma and multiple myeloma cell lines. One of the most important study focusing alendronate conjugated drug

delivery system is revealed by Ronit et all. They developed a novel approach to target bone metastasis by demonstrating the conjugation of Paclitaxel and Alendronate with N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer. The resulting copolymer PTX–FK–ALN is very effective in terms of cytotoxicity and antiangiogenecity [49]. After that they demonstrated the antitumor and antiangiogenic activity of the polymer conjugate via in-vivo and pharmacokinetic studies [50].

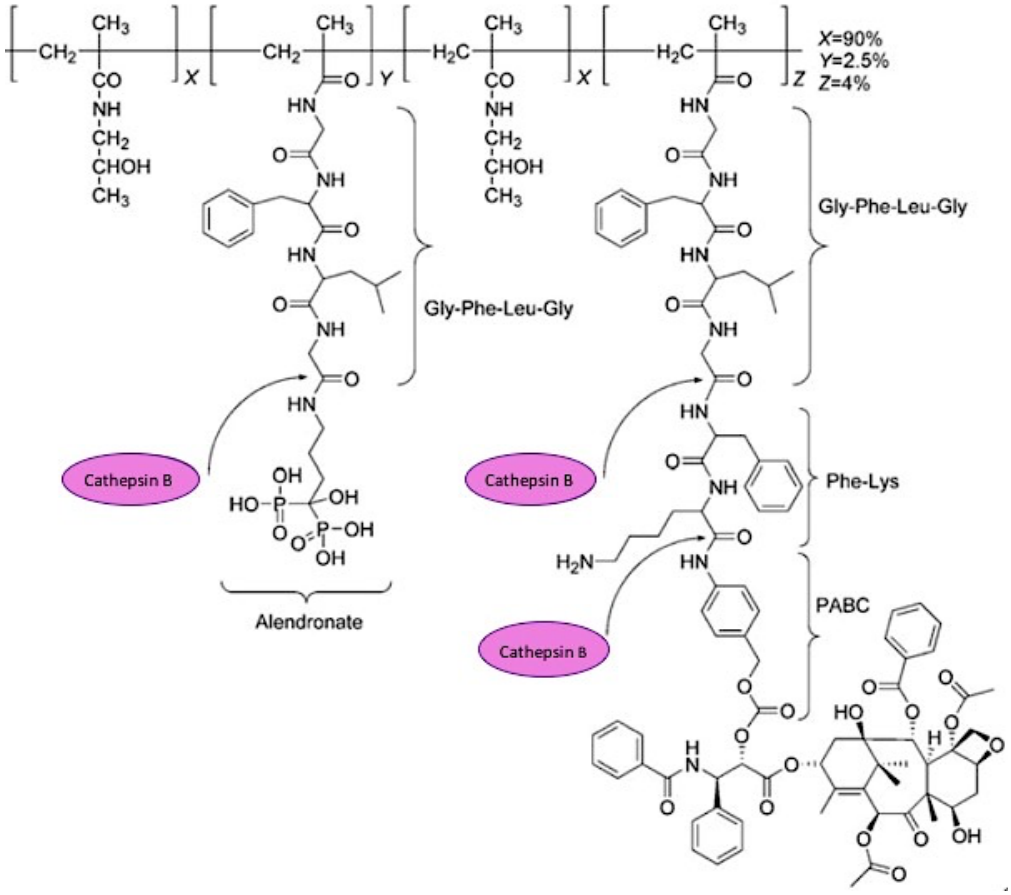


Figure 1.13. Chemical structure of the cleavable HPMA copolymer–PTX–FK–ALN [17].

Another remarkable study focusing alendronate conjugated drug delivery system addressing many of the problems encountered with conventional chemotherapy is revealed by Sanyal et all. In this work, they prepared and evaluated poly(oligoethylene glycol)methacrylate based polymers bearing an antiangiogenic drug combretastatin A4 (CA4) and alendronate for bone cancer. In order to understand and prove the targeting capacity of drug combretastatin A4 (CA4) and alendronate bearing polymer conjugate, they

investigated the binding potency to HA molecule. For this aim, they designed and synthesized targeted (a) and non-targeted (b) polymer conjugates to explore binding role of alendronate when it binds to polymer scaffold with CA4. Molecules were evaluated to demonstrate their bone targeting efficiency and cytotoxicities against endothelial cells.

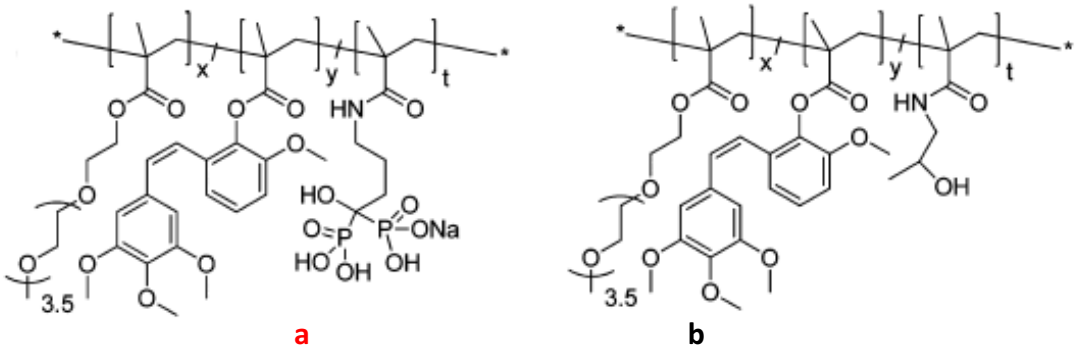


Figure 1.14. Structure of targeted P(OEGMA-CA4-ALN) (a) and non-targeted P(OEGMA-CA4) (b) polymer conjugates [45].

Targeted (a) and non-targeted (b) polymer conjugates were incubated with HA and binding percentage of each polymer with HA was evaluated at certain time points. According to the binding kinetics result, 75% of targeted (a) molecule was bound to HA, while it is observed for non-targeted (b) polymer as 3% after 60 min of incubation (Figure 1.15) [45].

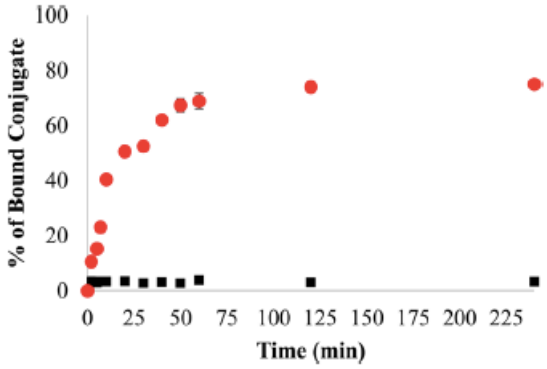


Figure 1.15. Binding kinetics of P(OEGMA-CA4-ALN) (a) (red circle) and P(OEGMA-CA4) (b) (black square) copolymers to bone mineral HA [45].

2. AIM OF THE STUDY

The aim of this study is to synthesize multifunctional and well-defined novel polymer bearing activated carbonate groups on the side chain and develop their application regading to functionalization with an amine containing drug molecule to prepare polymer-drug conjugates. Poly(oligo(ethylene glycol) methacrylate) (PEGMEMA)-based hydrophilic polymers incorporating NHS-activated carbonate group bearing side-chains were synthesized by using reversible addition fragmentation chain transfer (RAFT) polymerization due to make functional polymers with precise molecular weight and lower polydispersity. It is very important to design hydrolytically cleavable linker in order to control drug release under physiological condition, in accordance with this purpose carbamate-linked containing drug conjugate is designed. Alendronate is used as a targeting group because of its affinity to hydroxyapatite which is bone mineral. Cellular targeting, release profile and hydroxyapatite binding experiments demonstrates the feasibility of polymer-drug conjugate.

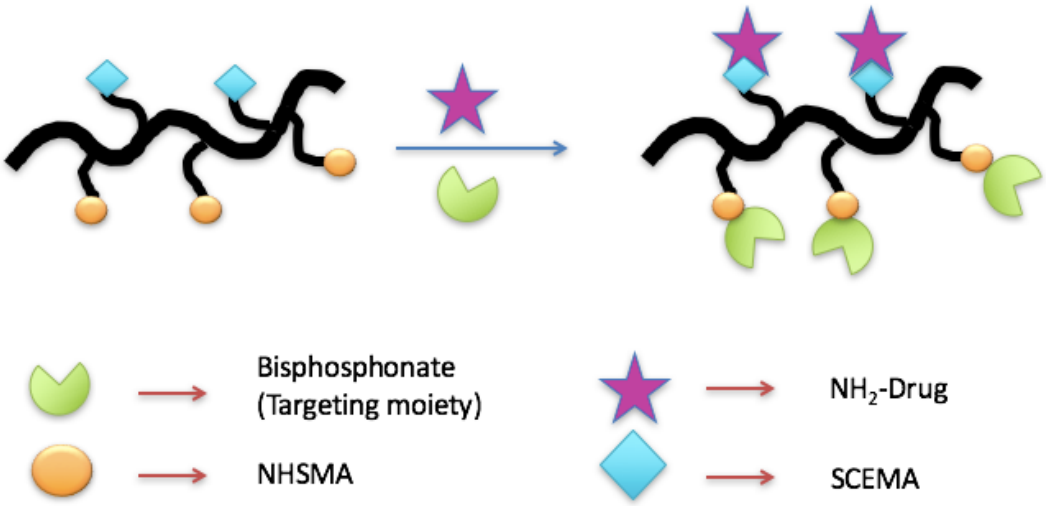


Figure 2.1. General scheme of the project.

3. EXPERIMENTAL

3.1. Materials and Characterization

Poly(ethylene glycol) methyl ether methacrylate (PEGMEMA, M_n=300) was purchased from Sigma Aldrich and purified by passing through an activated aluminum oxide column prior to use. 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid triethylamine was purchased from Sigma Aldrich and used as received. 2,2′-Azobis(2-methylpropionitrile) (AIBN) was purchased from Sigma Aldrich and recrystallized from methanol before use. Anhydrous sodium sulfate, dichloromethane, hexane, ethyl acetate, dioxane and methanol were purchased from Merck. N-hydroxysuccinimide-based carbonate monomer (SCEMA) and N-hydroxysuccinimide methacrylate (NHSMA) were prepared according to previously reported literature protocols [32]. Doxorubicin hydrochloride (DOX) was obtained from Sigma Aldrich. The molecular weights were determined by gel permeation chromatography analysis using Shimadzu RID-10A. DMAC was used as eluent at a flow rate of 1 mL/min at 30 C. PSS WinGPC software was used to process data. Characterization of compounds and copolymers were performed using ^{1}H NMR and ^{31}P spectroscopy (Varian 400 MHz). Drug release and binding studies were performed with a Varian Cary 50 Scan UV/vis and Fluorescence spectrophotometer.

3.2. Copolymerization of SCEMA and NHSMA with PEGMA via RAFT Polymerization

2-(N-succinimidylcarboxy) ethyl methacrylate (SCEMA) (130.0 mg, 0.48 mmol), N-hydroxysuccinimide methacrylate (NHSMA) (44 mg, 0.24 mmol), poly(ethylene glycol) methyl ether methacrylate (PEGMEMA) (500 mg, 1.6 mmol), 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid (CTA) (0.56 mg, 0.002 mmol) and AIBN (0.013 mg, 0.00008mmol) were dissolved in dioxane (3.50 mL) and placed in a sealed round-bottom flask equipped with a magnetic stir bar. The reaction mixture was sealed and purged with N_2 for 15 min and heated at 80 °C for 1 h. At the end of 1 h, the reaction temperature was decreased to 60 °C and stirred at this temperature for 23 h. After polymerization, the solvent was removed under *vacuo* and the residue was redissolved in dichloromethane (1

mL) and precipitated in cold Et_2O. The precipitated copolymer was dried under *vacuo* and characterized using SEC and and ^{1}H NMR. Mn = 48 kDa, Mw/Mn = 1.5 (75 % yield). ^{1}H NMR ($CDCl_3$, δ, ppm) 4.56 (br s, 2H, C*H*$_2$OCOO), 4.21 – 4.06 (br s, 4H, COO-C*H*$_2$) 3.65 (br s, 14H, (-C*H*$_2$C*H*$_2$O-), 3.52 (br s, 2H, C*H*$_2$CH$_2$O), 3.37 (s, 3H, O-C*H*$_3$), 2.87-2.80 (br d, 8H succinimide *H* at SCEMA and NHSMA), 1.74 – 0.82 (m, C*H*$_2$ and C*H*$_3$ along polymer backbone).

3.3. Orthogonal Functionalization of NHSMA/SCEMA copolymer with Doxorubicin Conjugation

NHSMA and SCEMA functional groups bearing PEGMA copolymer (50.0 mg, 0.001 mmol) was dissolved in dry dichloromethane (2 mL). Doxorubicin hydrochloride (29.0 mg, 0.050 mmol) and triethylamine (9.0 μL, 0.064 mmol) was then added to this solution and the reaction mixture was stirred at 0 °C for 2 h. After dialysis using 3.5 kDa cutoff regenerated cellulose acetate membrane in acetonitrile (200mL) for 24 h, viscous and red copolymer 5 was obtained by lyophilization. ^{1}H NMR ($CDCl_3$, δ, ppm) 7.95 – 7.76 (br d,s 3H, –CH=CHCH=), 4.28 – 4.09 (br s, 4H, COO-C*H*$_2$), 3.63 (br s, 14H, (-C*H*$_2$C*H*$_2$O-), 3.52 (br s, 2H, C*H*$_2$CH$_2$O), 3.37 (s, 3H, O-C*H*$_3$), 2.81 (br s, 4H, succinimide *H* at NHSMA) 1.73 – 0.82 (m, C*H*$_2$ and C*H*$_3$ along polymer backbone).

3.4. Alendronate Conjugation of Copolymer 5

Copolymer 5 containing DOX and NHSMA molecules (20 mg, 0.0004) was dissolved in acetonitrile (0.2 mL). Alendronate (6 mg, 0.018 mmol) was dissolved in distilled water (0.3 mL) and set the pH value of the reaction mixture was maintained at 8.5 by dropwise addition of 0.2 mol/L NaOH / EDTA solution. Alendronate solution was added into the first polymer solution dropwise and the reaction mixture was stirred at 45 °C for 4 days. After the reaction, the product was dialyzed using 3.5 kDa cutoff regenerated cellulose acetate membrane in water (200mL) for 24 h to obtain purified copolymer 6. ^{1}H NMR ($CDCl_3$, δ, ppm) 7.95 – 7.71 (br d,s 3H, –*CH*=*CH*-*CH*=), 4.28 – 4.09 (br s, 4H, COO-C*H*$_2$), 3.63 (br s, 14H, (C*H*$_2$C*H*$_2$O-)3.5), 3.52 (br s, 2H, C*H*$_2$CH$_2$O), 3.35 (s, 3H, O-C*H*$_3$), 2.63 (br s, 2H, *CH*$_2$-NH-) 1.73 – 0.82 (m, C*H*$_2$ and C*H*$_3$ along polymer backbone).

3.5. Copolymerization of SCEMA with PEGMA Monomers via RAFT Polymerization

2-(N-succinimidylcarboxy)ethyl methacrylate (SCEMA) (60.0 mg, 0.22 mmol), poly(ethylene glycol) methyl ether methacrylate (PEGMEMA) (200 mg, 0.66 mmol) 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (CTA) (1.15 mg, 0.004 mmol) and AIBN (0.14 mg, 0.0008mmol) were dissolved in dioxane (1.25 mL) and placed in a sealed round-bottom flask equipped with a magnetic stir bar. The reaction mixture was purged with N_2 for 15 min and stirred at 70 °C for 18 h. After polymerization, the solvent was removed under *vacuo* and the residue was redissolved in dichloromethane (1 mL) and precipitated in cold Et_2O. The precipitated copolymer 7 was dried under *vacuo* and characterized using SEC and and ^{1}H NMR. Mn = 33 kDa, Mw/Mn = 1.3 (70 % yield). ^{1}H NMR ($CDCl_3$, δ, ppm) 4.55 (br s, 2H, C*H*$_2$OCOO), 4.22 – 3.79 (m, 4H, COO-C*H*$_2$) 3.63 (br s, 14H, (-C*H*$_2$C*H*$_2$O-), 3.52 (br s, 2H, -C*H*$_2$CH$_2$O-), 3.34 (s, 3H, O-C*H*$_3$), 2.88 (s, succinimide *H*), 1.89 – 0.82 (m, C*H*$_2$ and C*H*$_3$ along polymer backbone)

3.6. Sequential Functionalization of SCEMA Copolymer with Doxorubicin and Alendronate

SCEMA group bearing PEGMA copolymer (50.0 mg, 0.0015 mmol) was dissolved in dry dichloromethane (2 mL). Doxorubicin hydrochloride (12.0 mg, 0.021 mmol) and triethylamine (6.0 μL, 0.043 mmol) was then added into this solution under N_2 atmosphere and the reaction mixture was stirred at 0 °C for 2 h. Afterward the solvent was removed under *vacuo* and the residue was dissolved in acetonitrile (0.5 mL). Alendronate (8 mg, 0.024 mmol) was dissolved in milli-q distilled water (0.75 mL) and set the pH value of the reaction mixture was maintained at 8.5 by dropwise addition of 0.2 mol/L NaOH/EDTA solution. Alendronate solution was added into the first polymer solution dropwisely and the reaction mixture was stirred at 8 °C for 4 h then warmed to RT for 15 h. After the reaction, the product was dialyzed using 3.5 kDa cutoff regenerated cellulose acetate membrane in distilled water (200mL) for 24h to obtain purified copolymer 8 (74 % yield). ^{1}H NMR ($CDCl_3$, δ, ppm) 7.92 – 7.72 (br d,s 3H, –CH=CHCH=), 4.28 – 4.09 (br s, 4H, COO-C*H*$_2$), 3.63 (br s, 14H, (-C*H*$_2$C*H*$_2$O-), 3.52 (br s, 2H, C*H*$_2$CH$_2$O), 3.37 (s, 3H, O-C*H*$_3$), 2.63 (br s, 2H, –CH$_2$–NH-) 1.73 – 0.82 (m, C*H*$_2$ and C*H*$_3$ along polymer backbone).

3.7. Doxorubicin Conjugation of SCEMA Copolymer

SCEMA functional group bearing PEGMA copolymer (50.0 mg, 0.001 mmol) was dissolved in dry dichloromethane (2 mL). Doxorubicin hydrochloride (14.0 mg, 0.024 mmol) and triethylamine (7.0 μL, 0.064 mmol) was then added to this solution and the reaction mixture was stirred at 0 °C for 2 h. After dialysis using 3.5 kDa cutoff regenerated cellulose acetate membrane in acetonitrile (200mL) for 24 h, viscous and red copolymer 5 was obtained by lyophilization. ^{1}H NMR ($CDCl_3$, δ, ppm) 8.01 – 7.79 (br d,s 3H, –CH=CHCH=), 4.28 – 4.09 (br s, 4H, COO-C*H*$_2$), 3.63 (br s, 14H, (-C*H*$_2$C*H*$_2$O-), 3.52 (br s, 2H, C*H*$_2$CH$_2$O), 3.38 (s, 3H, O-C*H*$_3$), 1.73 – 0.82 (m, C*H*$_2$ and C*H*$_3$ along polymer backbone).

3.8. In Vitro Drug Release Experiment of Copolymer 8

The obtained DOX and alendronate ALN containing copolymer 8 was dissolved in acetate buffer (pH 5.4) and phosphate buffered saline (PBS, pH 7.4) solutions (2 mL), sealed in 3.5 kDa cutoff regenerated cellulose acetate membrane dialysis bag. The dialysis bags were incubated in release mediums (20 mL) at 37 °C under 120 rpm oscillation. The solution outside the dialysis bag was removed from the release medium within predetermined time intervals and replaced with same volume of fresh buffer solutions. The amount of drug in collected media was determined spectrophotometrically using the emission of doxorubicin at 585 nm with the help of a calibration curve. The results were expressed in terms of cumulative release as a function of time.

3.9. Binding Kinetics of Copolymer 8 with HA

The binding kinetics of copolymer 8 with HA was investigated in this step. Hydroxyapatite (100 mg) were incubated in phosphate-buffered saline (PBS) (4 mL) with the same doxorubicin (0.1 mg) concentration of each copolymer 8 and copolymer 9 in a falcon tube. As a reference (i.e., 0% binding), the samples were incubated in falcon tubes without HA mineral. Each mixture was gently shaken at 37 °C in water bath. After 0, 5, 10, 30, and 60 minute each mixture solution was centrifuged (9000 rpm, 3 min) and the absorbance of the supernatant was measured using the absorbance of doxorubicin at 233 nm

by UV/vis spectrophotometer. The HA binding percentage was calculated as by the below formula.

$$\% \text{ Binding} = \frac{A_{(\text{Copolymer})_0} - A_{(\text{Copolymer})_t}}{A_{(\text{Copolymer})_0}} \times 100$$

4. RESULTS AND DISCUSSION

4.1. Synthesis of Orthogonally Functionalizable Copolymer

Functional polymers are very promising scaffolds in wide range areas such as conjugated drug delivery, coating and micellar systems. On the other hand, chemoselective reactions are very important due to interference of the other functional groups' activity in the medium. Well-defined polymeric materials bearing activated esters can be obtained by using various free radical polymerization techniques. In this study, the orthogonal functionalization of two different NHS-activated amine reactive groups with hydrophilic PEGMA monomers is done via reversible addition fragmentation chain transfer (RAFT) polymerization. Orthogonal functionalization provides an opportunity to selectively attach same functional moiety such as targeting group and active drug agent. In drug delivery applications, hydrophilicity and biocompatibility of polymer constructs is the first requirement for such applications.

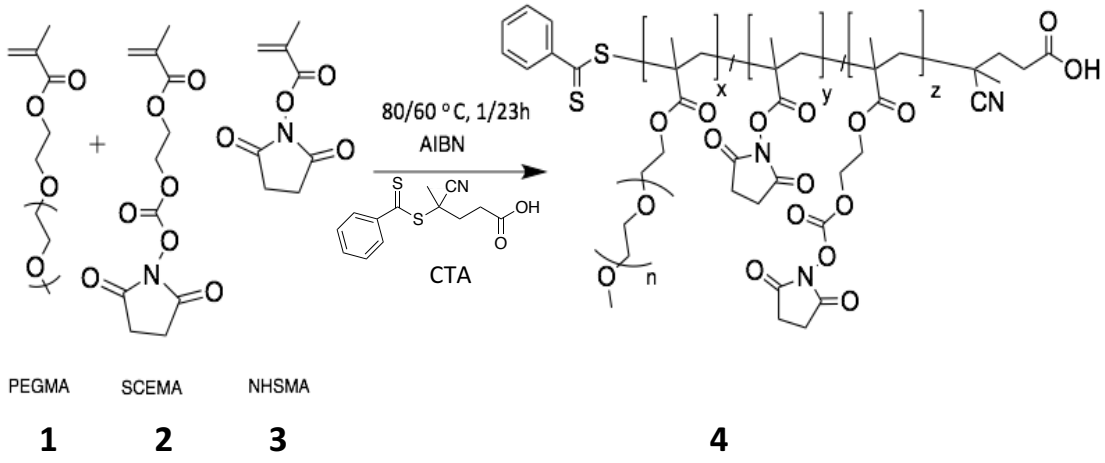


Figure 4.1. Synthesis of SCEMA/NHSMA Copolymer 4 via RAFT Polymerization.

SCEMA monomer was synthesized according to previous published study [32]. NHS-activated carbonate and ester group bearing potential drug delivery system was synthesized via RAFT polymerization of SCEMA and NHSMA with hydrophilic PEGMA monomers in the presence of AIBN initiator. Polymer was obtained in pure form after

removal of unreacted monomers upon their precipitation with Et_2O. The resulting copolymer was obtained with 1.5 narrow polydispersity (PDI) and 48 kDa high molecular weight according to SEC analysis. The incorporation ratio of SCEMA and NHSMA monomers onto the polymer backbone was evaluated by the relative integration of the peaks at 2.80-2.87 ppm comes from succinimide parts of these compounds. Due to the partial overlapping of these peaks at NHS site, -CH_2OCOO protons of SCEMA at 4.56 ppm was also evaluated in order to understand the incorporation ratio of each compound individually.

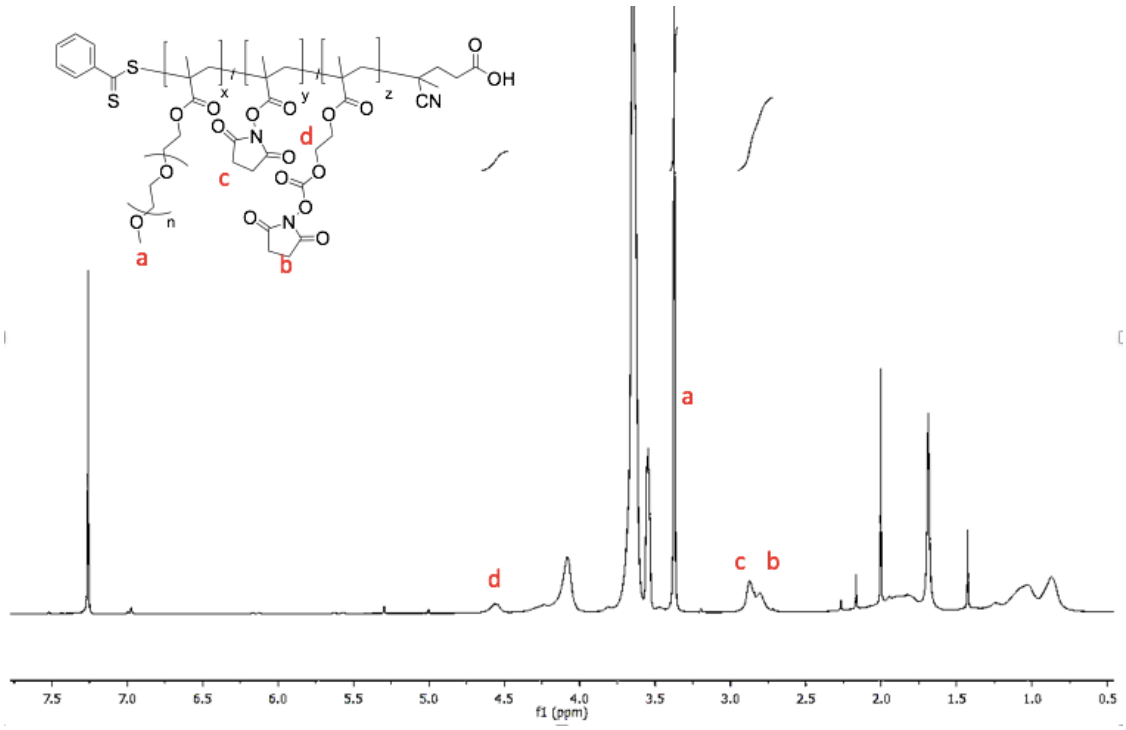
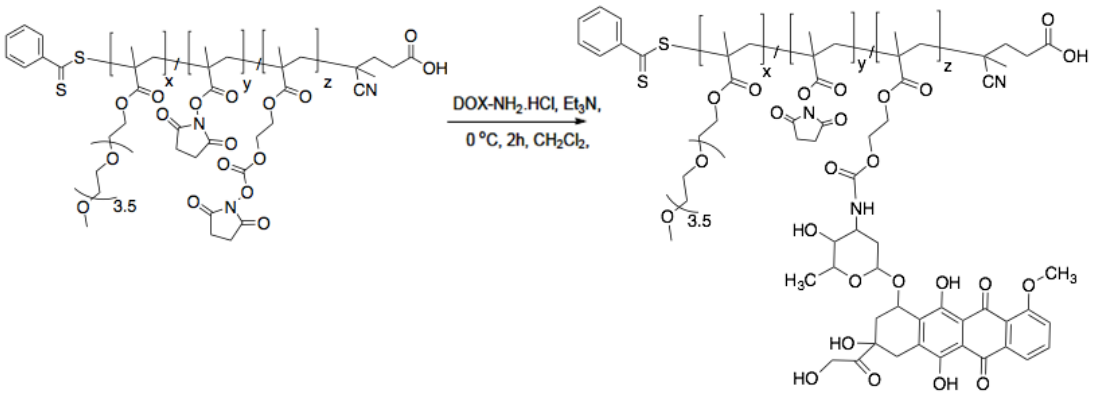


Figure 4.2. Copolymerization of SCEMA and NHSMA with PEGMA Monomers via RAFT Polymerization.

The obtained functional polymeric material provides an opportunity to selectively attach to drug containing reactive amine to the NHS-activated carbonate monomer thanks to the reactivity of SCEMA. On the other hand, due to the orthogonally functionalizable feature of the polymer, amine group containing targeted group can bind to the polymer after drug attachment.

4.2. Orthogonal Functionalization of Amine Reactive Copolymer with Amine Containing Drug

Orthogonally functionalizable feature of NHSMA and SCEMA containing copolymer 4 was re-proved using amine containing chemotherapeutic drug doxorubicin. In this copolymer both of the reactive groups can react with the amines but the resulting covalent bonding is different. Amine conjugation of carbonate side chain results in a carbamate linkage which is a hydrolyzable linker. But same conjugation with NHSMA results in amide bond which is hydrolytically stable linkage. All of these features make these functional groups bearing polymer as a suitable candidate for both attachment of drug and targeting moiety.



4 **5**

Figure 4.3. Functionalization of SCEMA and NHSMA containing copolymer 4 with doxorubicin.

The excess amount of DOX was used for quantitative conjugation with SCEMA containing copolymer via carbamate formation. To ensure efficient functionalization and neutralization of DOX-NH_2.HCl, small amount of triethylamine was used. The incorporation ratio of Doxorubicin with the carbonate monomer was evaluated by the relative integration of the peaks belong to Doxorubicin, PEGMA and NHSMA. Quantitative functionalization of the polymer was proved by the means of the disappearance of proton resonance at 2.83 ppm due to the carbonate succinimide protons. Newly formed proton signals belonging to doxorubicin are another evidence for successful conjugation. On the

other hand, orthogonal functionalization of SCEMA and NHSMA containing copolymer was demonstrated by the fact that the signal retaining at 2.78 shows that another amine reactive NHSMA stays intact during doxorubicin binding. ^{1}H NMR integration of the peaks comes from DOX and NHSMA is showing that there are 18% DOX and 7% NHSMA in the obtained copolymer as percentage by weight.

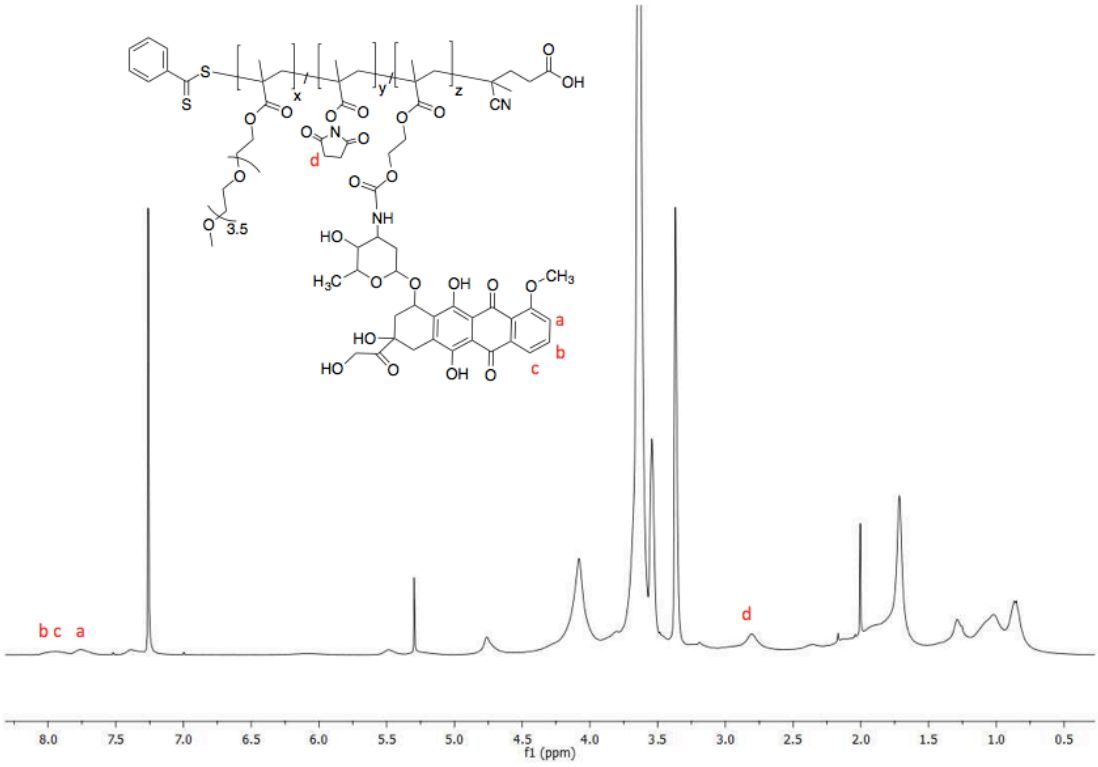


Figure 4.4. ^{1}H NMR spectrum of copolymer 5.

4.3. Alendronate Conjugation to Copolymer 5

Bisphosphonates inhibit hydroxyapatite breakdown, hence effectively prevent bone resorption [40]. Additionally, On the other hand, there are some evidences that are revealed by preclinical and clinical studies regarding to bisphosphonates also have antitumor activity which makes significant contribution to their therapeutic efficacy. This special property of bisphosphonates makes them considerable clinical agents also in drug delivery systems.

The main objective of this step is attachment of alendronate to the polymer backbone by non-hydrolyzable amide bond, utilising the HA affinity of alendronate molecule. The excess amount of alendronate was used for quantitative conjugation with NHSMA containing copolymer at 45 °C for 4 days.

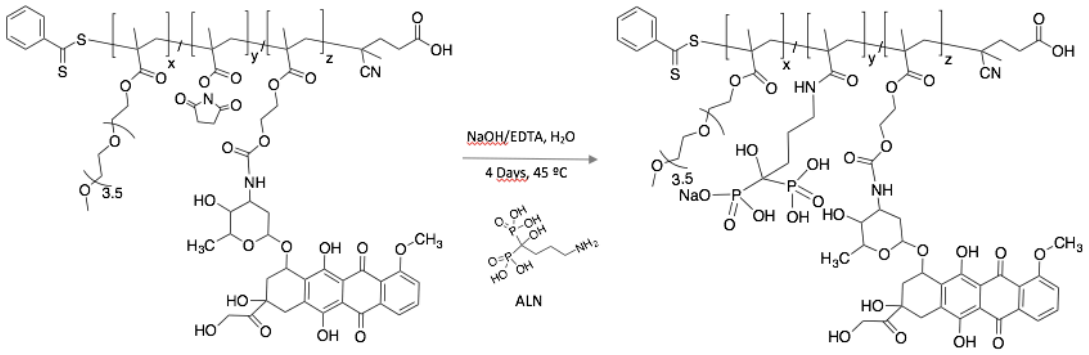


Figure 4.5. Alendronate conjugation of DOX containing copolymer 5

In addition to these reaction conditions, the pH value of the reaction was maintained at 8.5. ^{1}H NMR integration of the peaks comes from DOX and ALN is showing that there are 4% DOX and 1.5% ALN in the obtained copolymer 6 as percentage by weight. Despite the fact that small amount of alendronate attached to copolymer 5, there was only 4% DOX left from the starting material's doxorubicin ratio (16%). Hence almost 80% of DOX amount in the polymer conjugate went away after alendronate binding study compared to starting material's doxorubicin ratio. Although altering the temperature, time and pH value of binding conditions stepwise in different trials to solve breaking problems of DOX, the attachment of alendronate was successful only in this condition. In terms of drug yield onto the polymer, it is concluded that trying alendronate conjugation with different bond which is formed in mild conditions can be more effective and efficient compared to attachment via amide bond.

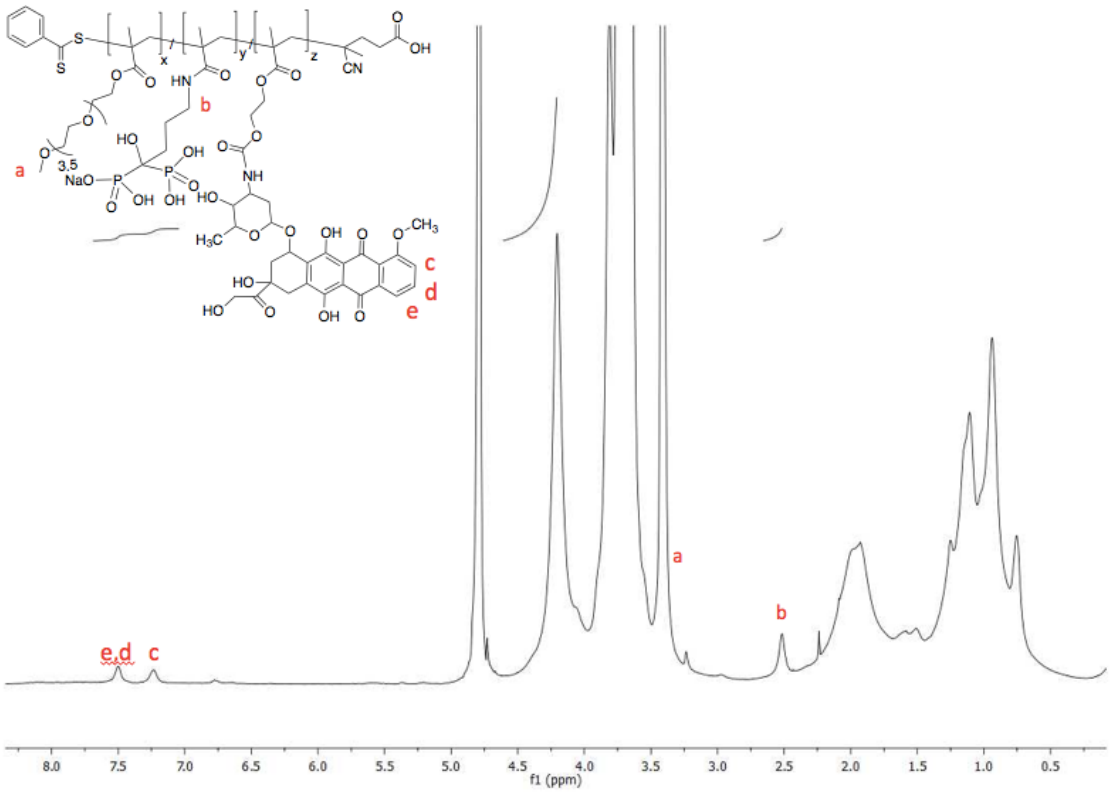


Figure 4.6. ^{1}H NMR spectrum of copolymer 6.

4.4. Copolymerization of NHS-activated Carbonate Group Containing Monomer (SCEMA) with PEGMA Monomers via RAFT Polymerization

For applications of polymers in the fields of biomedical sciences, especially in drug delivery, functionalization of polymeric materials draw considerable attention due to the fact it provides an opportunity to attach targeting groups along with drug molecules for targeted therapies.

Therapeutic agents and targeting moieties can be conjugated with polymeric materials using different chemical linkages such as carbamate, amide, ether and ester. It is very important to choose linkers for polymer–drug conjugates because the targeting group should remain on the polymer until fulfilling the targeting duty. When designing a polymer-drug conjugate bearing a targeting group which is linked to the polymer scaffold with hyrolyzable linker, it is very significant to research and prove the stability of the linker in plasma condition. Because of the great stability and cleavage conditions, carbamate linker

is important for polymer conjugated drug delivery systems [51]. In accordance with this purpose carbonate group bearing SCEMA copolymer is synthesized.

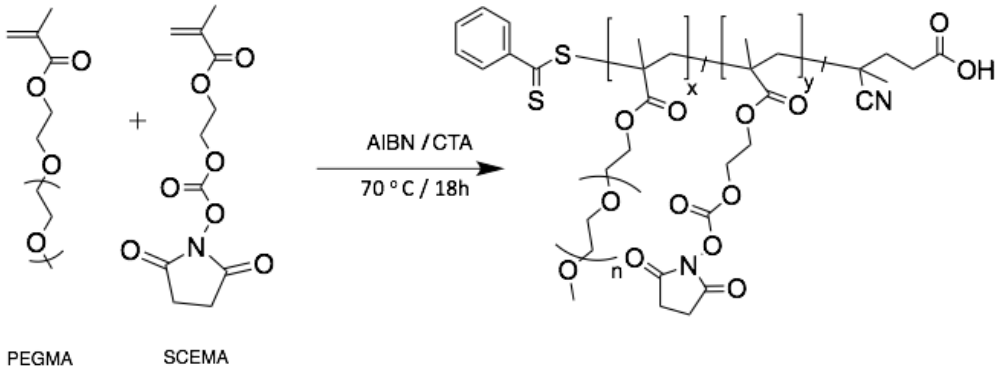


Figure 4.7. Synthesis of SCEMA Copolymer via RAFT Polymerization

NHS-activated carbonate group bearing potential drug delivery system was synthesized via RAFT polymerization of SCEMA with hydrophilic PEGMA monomers in dioxane at 70 ^{o}C. Polymer was obtained in pure form after removal of unreacted monomers upon their precipitation with Et_2O. The resulting copolymer was obtained with 1.3 narrow polydispersity and 33 kDa high molecular weight according to SEC analysis. The incorporation ratio of SCEMA monomer onto the copolymer 7 was evaluated by the relative integration of the peaks at 2.88 and 3.33 ppm belonging to succinimide protons of SCEMA and methoxy protons of PEGMA, respectively. According to the calculation based on integration of each related peaks, there is 22% SCEMA in the obtained copolymer 7 as percentage by weight.

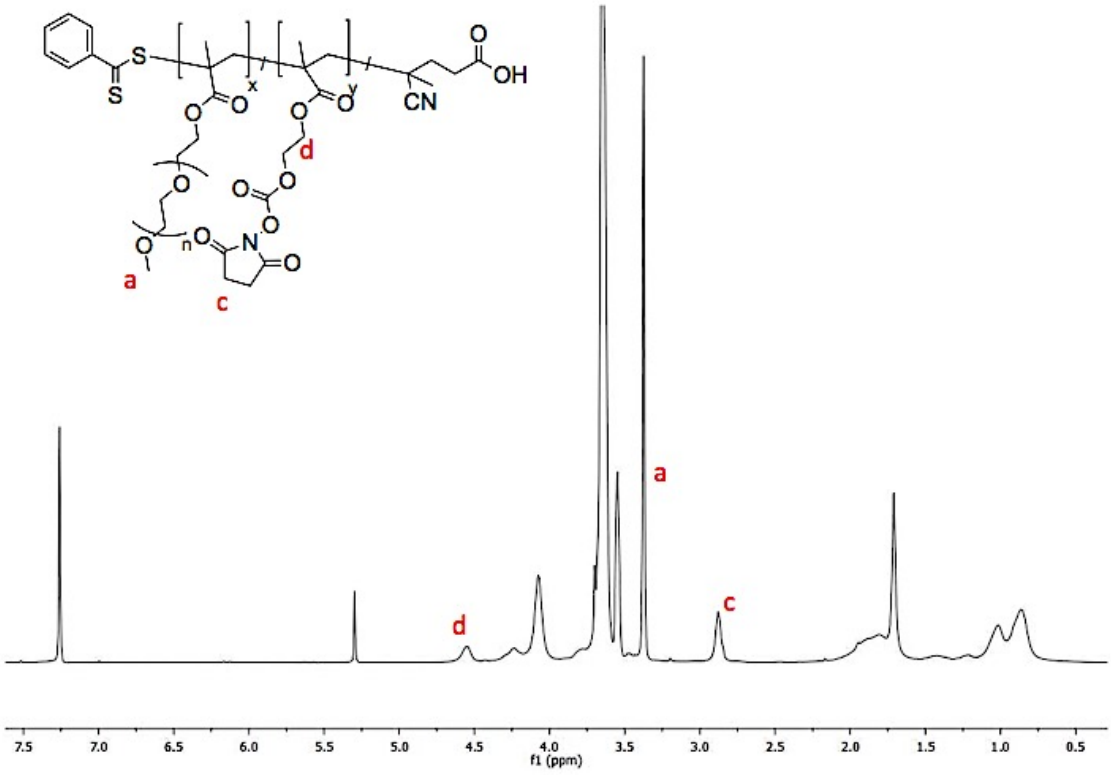


Figure 4.8. ^{1}H NMR spectrum of copolymer 7.

The obtained NHS-activated carbonate group bearing polymer has a great potential to attach amine group containing active drug agent and targeting group.

4.5. Sequential Drug and Targeting Group Conjugation of Copolymer 7

In the previous study, in order to obtain alendronate and doxorubicin containing copolymer drug conjugate, two different monomers which form hydrolyzable and non-hydrolyzable linkage was used but due to the difficult binding condition of alendronate, most of the doxorubicin amount on the polymer was released. According to result which is obtained from the previous study, it is very important to choose different linkage to bind alendronate which is taken place in mild conditions. Hence, it is concluded that choosing carbamate bond for alendronate binding onto the polymer can be an effective solution in terms of drug yield onto the polymer.

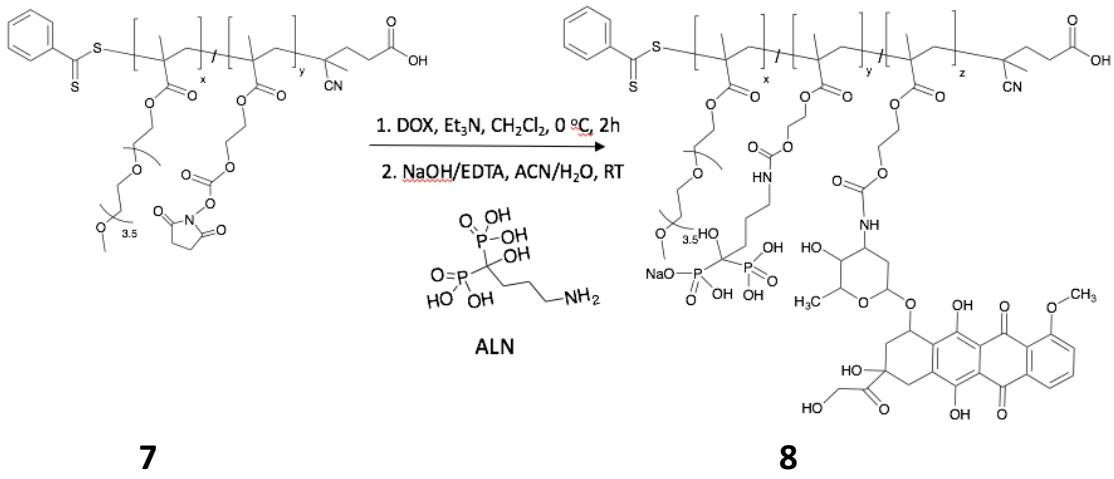


Figure 4.9. Sequential synthesis of SCEMA containing PEGMA copolymer with drug and targeting group via carbamate bond.

Carbonate containing functional monomers are one of the most suitable derivatives for binding of amine bearing drugs and targeting groups in order to form cleavable carbamate linker between drug or targeting moiety and the monomer. The stability of the drug-polymers linkage is very crucial under different biological conditions. This linkage possesses the inherent biodegradability property to release the payload, although it is very stable in the plasma. Based on the stability and cleavage conditions, the carbamate linkage has highly attracted attention in polymer therapeutics to bind both targeting group and drug.

Doxorubicin amount which was used for quantitative conjugation with half of the SCEMA onto the polymer was calculated by integration ratio of succinimide protons. To ensure efficient functionalization and neutralization of Dox-NH_2.HCl, small amount of triethylamine was used. The main objective of the upcoming step is attachment of alendronate to the polymer backbone via carbamate bond, utilising the HA affinity of alendronate molecule. The incorporation ratio of Doxorubicin alendronate with the carbonate monomer was evaluated by the relative integration of the peaks belong to doxorubicin, alendronate, PEGMA and NHSMA. Newly formed proton signals at 7.95 – 7.71 and 2.63 ppm belonging to doxorubicin and alendronate respectively, are other evidences for successful conjugation. The resulting polymer conjugate contains 8% doxorubicin and 1.5% alendronate by weight percentage for targeting and therapeutic aim.

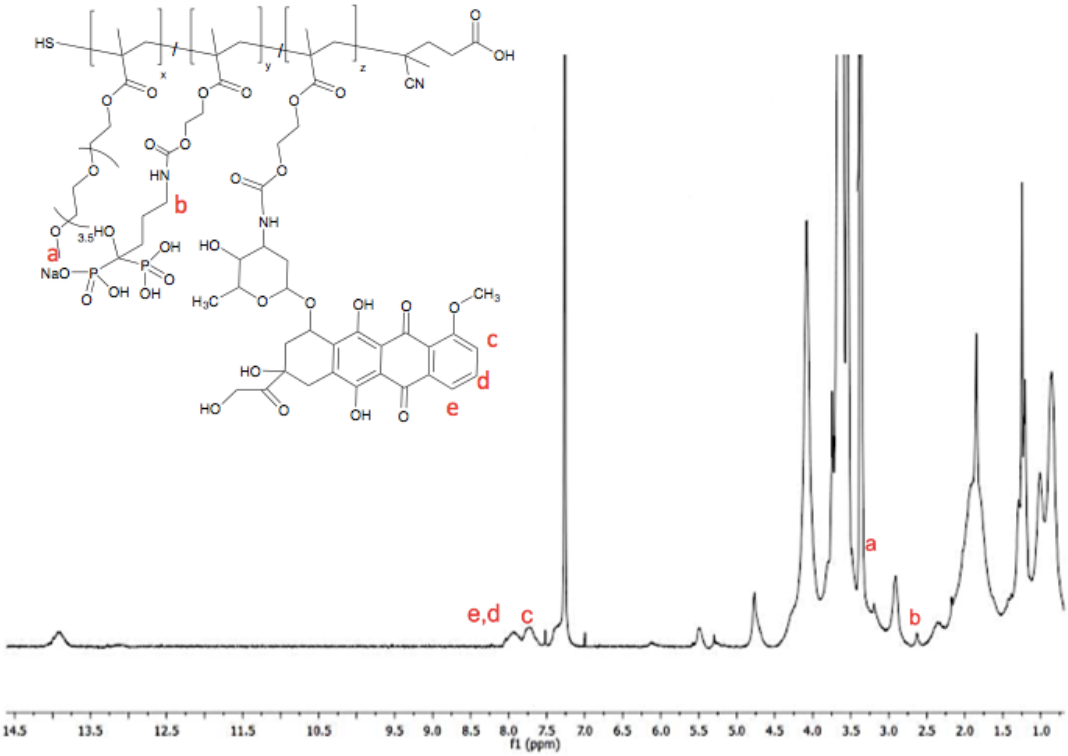


Figure 4.10. ^{1}H NMR spectrum of Copolymer 8.

4.6. Doxorubicin Conjugation of SCEMA Copolymer

Alendronate is very effective compound in terms of bone targeting due to their exceptional high affinity to HA mineral in bone tissue. In order to understand and prove the binding role of ALN in the obtained copolymer 8, it is very important to compare the binding capacity between a molecule containing only the same drug conjugated molecule without ALN. For this aim, copolymer 9 was synthesized as illustrated in figure 4.11. According to the ^{1}H NMR data of copolymer 9, there is 7% DOX on the polymer in terms of percentage of weight.

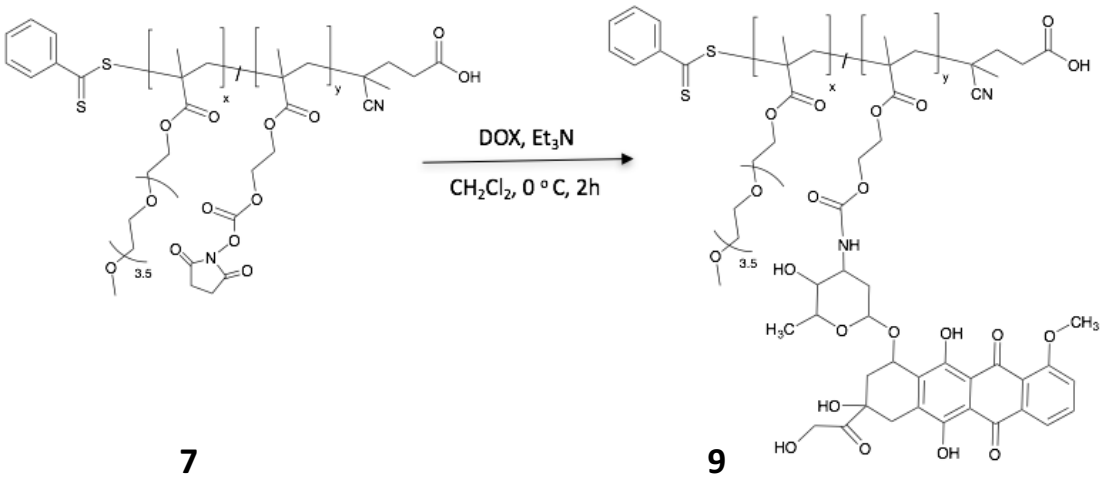


Figure 4.11. Doxorubicin conjugation of SCEMA copolymer.

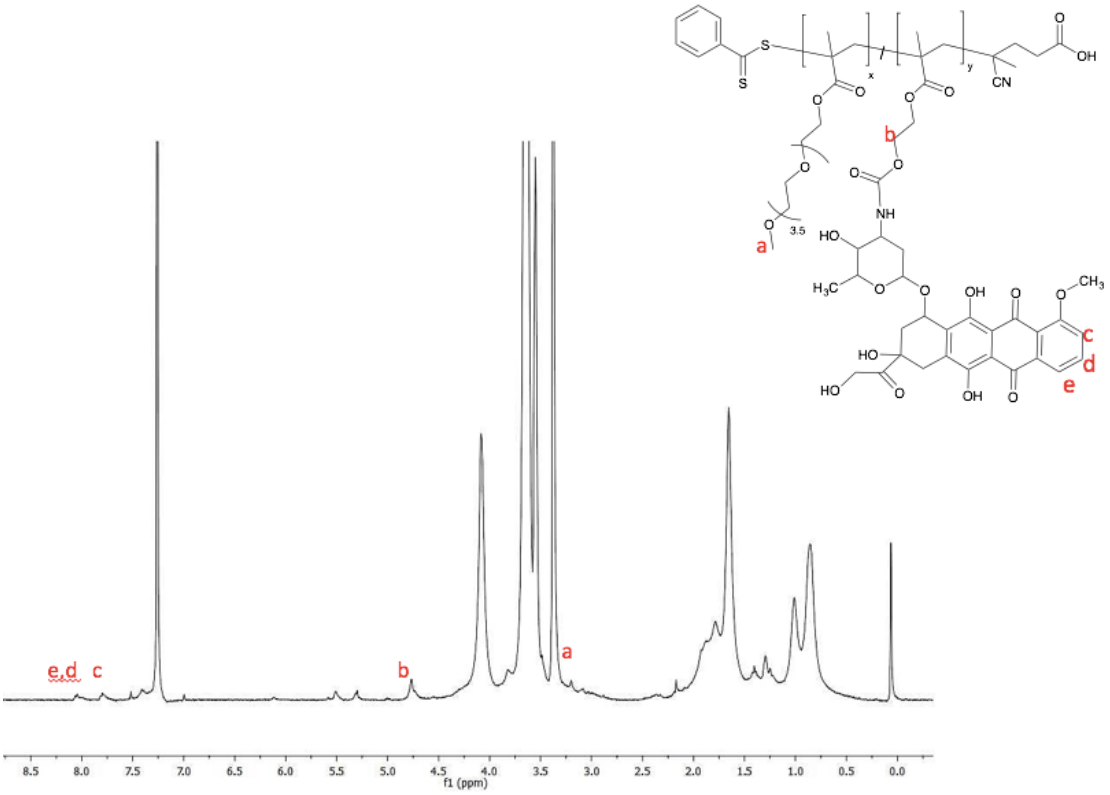


Figure 4.12. ^{1}H NMR spectrum of copolymer 9.

4.7. In-vitro Drug Release

This study demonstrated the synthesis and characterization of a polymer-drug conjugate with functionalizable amine reactive moiety as a drug delivery system. In this sense, the other significant part of the platform after synthesis is demonstrating the feasibility of the polymer-drug conjugate in biological conditions by mimicking plasma and tumor medias. Thanks to the activated carbonate side groups of the copolymer, we synthesized the chemotherapeutic drug doxorubicin conjugated polymer platform via hydrolytically cleavable carbamate bond. By utilizing the activated carbonate side groups of polymer, we hypothesized that the conjugate would release its active drug agent by the means of slow breaking of carbamate bond.

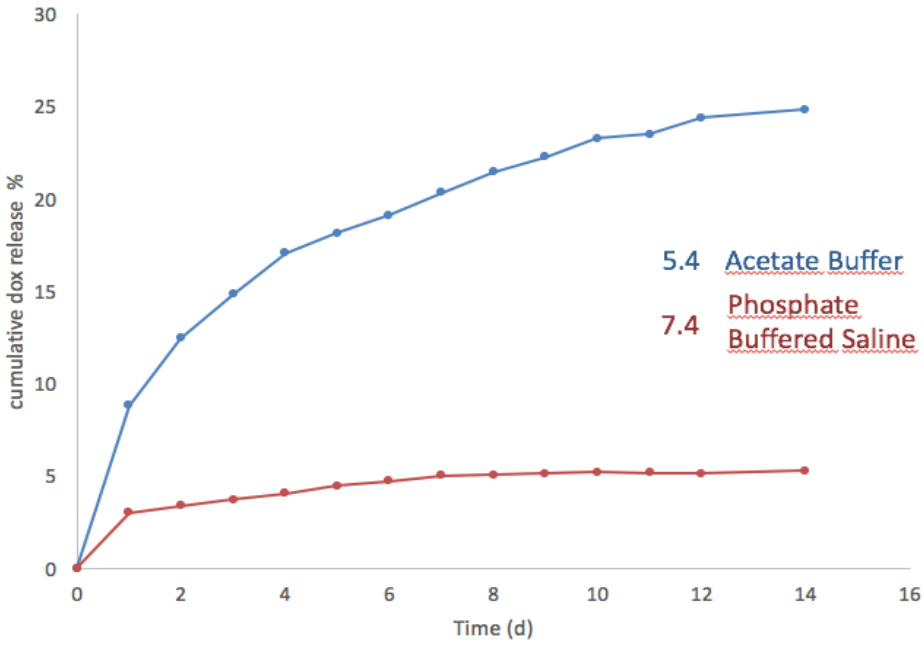


Figure 4.13. In vitro doxorubicin release from targeted polymer-drug conjugates.

The obtained copolymer 8 was investigated in in vitro environment in order to understand the amount of released drug in two different biological conditions. Copolymer 8 that are subject to drug release via hydrolysis of carbamate bonds is incubated in acetate buffer (pH 5.4) and phosphate buffered saline (PBS, pH 7.4) solutions to observe in vitro behavior. Figure 4.13 illustrates the release trend of the resulted polymer-drug conjugate in two different medias. There is no initial burst release which was observed based on the first

data point of the study. This result can be an evidence for chemical attachment of DOX molecules to the polymer scaffold by eliminating physical release from the polymer. It is expected that higher drug release was observed with the lower pH value whereas in PBS, dramatically lower amount of drug was released from the polymer conjugate. Thus, slow, sustained and improved degradation of carbamate bond was observed in acidic environment for 2 weeks.

4.8. Hydroxyapatite (HA) Binding Assay

One of the main constituent of skeleton hydroxyapatite was exposed in lysis site, for the metastasized or invaded bone tissue. It is widely documented with lots of literature examples, bisphosphonates have strong affinity with bone tissue due to its hydroxyl and phosphonate groups. It is very important to estimate binding capacity of the targeted polymer conjugate to prove the targeting efficiency. In accordance with this purpose hydroxyapatite binding assay was carried out in vitro.

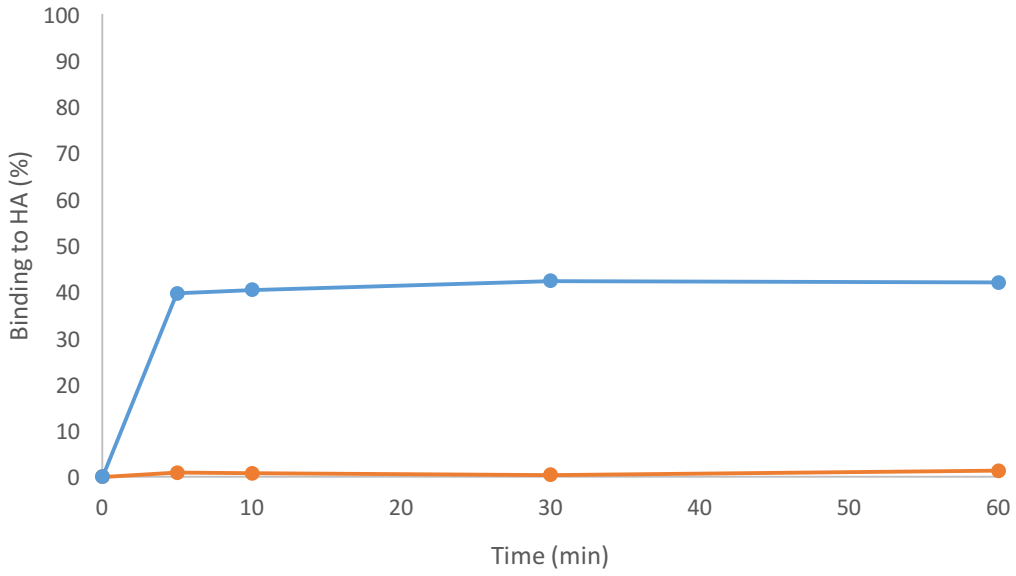


Figure 4.14. Binding kinetics of copolymer 8 (blue) and 9 (orange) with HA.

The binding kinetics of copolymer 8 and copolymer 9 with hydroxyapatite is showed in Figure 4.14. After 0, 5, 10, 30, and 60 minute, absorbance of the supernatant was measured using the absorbance of doxorubicin at 233 nm by UV/vis spectrophotometer.

Each absorption value of the incubated samples was recorded and the binding percentage was calculated based on the formula which is given the experimental section. After 5 min of incubation 40% of DOX containing targeted polymer conjugate was bound with hydroxyapatite. In contrast, as a control study, same experiment was done with the polymer conjugate which does not has any targeting group on its backbone named copolymer 9. According to the study result, 40% of the targeted polymer conjugate was bound with hydroxyapatite whereas almost any binding was observed in copolymer 9. The results indicate that the targeted copolymer 8 can precisely and quickly target the bone tissue lysis site.

On the other hand, the other proof for HA affinity of alendronate containing conjugate is color of the each precipitated HA crystals. Color of precipitate belongs to copolymer 8 is more reddish than copolymer 9 due to precipitation of alendronate bearing conjugate with HA (Figure A.8.).

5. CONCLUSION

Hydrophilic PEGMA based polymeric carrier was copolymerized with carbonate and active ester containing molecules via RAFT polymerization in high-molecular weight. The obtained copolymer was conjugated to the drug agent via carbamation of the amine group on doxorubicin molecule. As a bone targeting unit, a small molecule amino bisphosphonate drug alendronate was conjugated to the drug-containing copolymer via amidation. At the end of this reaction, the drug amount on the copolymer was decreased by 80% due to harsh condition of amidation step. In terms of drug yield on the polymer, it is concluded that trying alendronate conjugation via carbamate bond which is formed in milder conditions can be more efficient instead of amide bond. In accordance with this purpose, PEGMA based and carbonate group containing copolymer was synthesized and conjugated with doxorubicin and alendronate sequentially via carbamate linkers. On the other hand, in order to show the role of targeting group, PEGMA based drug containing copolymer was synthesized devoid of any targeting group. A remarkably preferential affinity to the bone mineral hydroxyapatite was observed for the bisphosphonate targeting group bearing polymer-drug conjugate, when compared to the copolymer devoid of any targeting unit. Effective release behavior of alendronate and doxorubicin functionalized PEGMA copolymer was investigated in biological conditions by mimicking plasma and tumor medias. According to the in-vitro drug release study, slow, sustained and pH depandant degradation of carbamate bond was observed in designed polymer platform. This bisphosphonate functional groups bearing polymer-drug conjugates can be further evaluated for cytotoxicity studies for metastatic bone cancer.

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APPENDIX A: SUPPORTING DATA

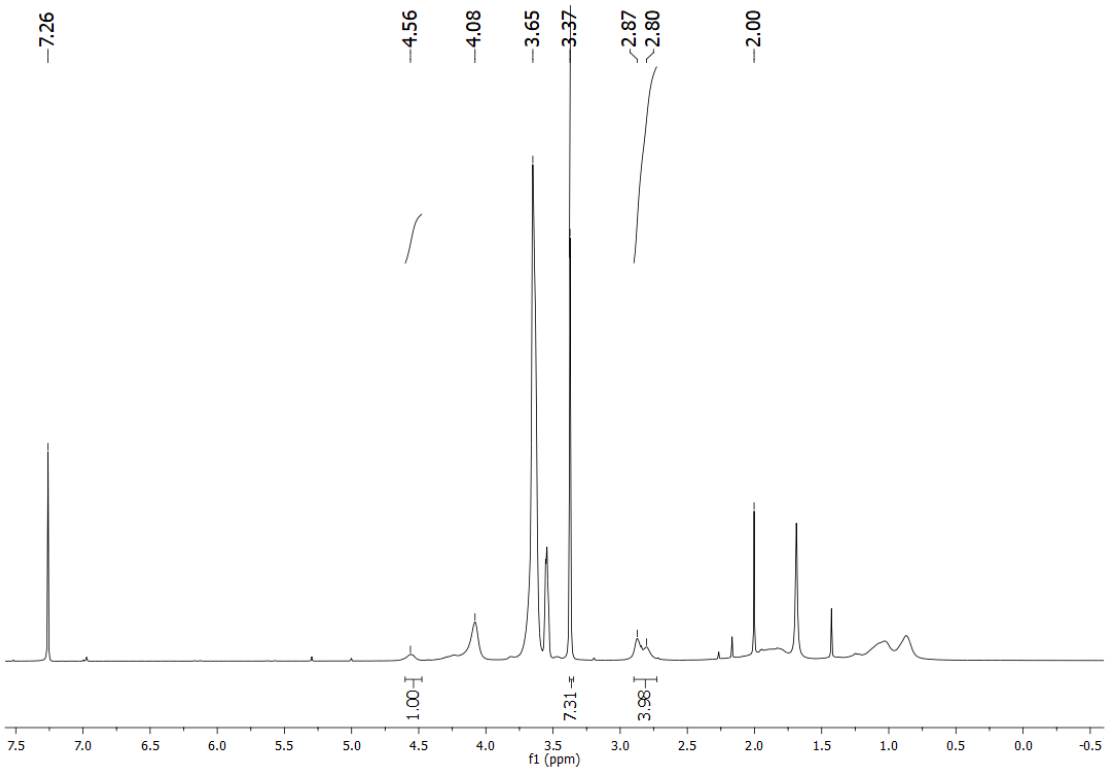

Figure A. 1. ^{1}H NMR spectrum of copolymer 4.

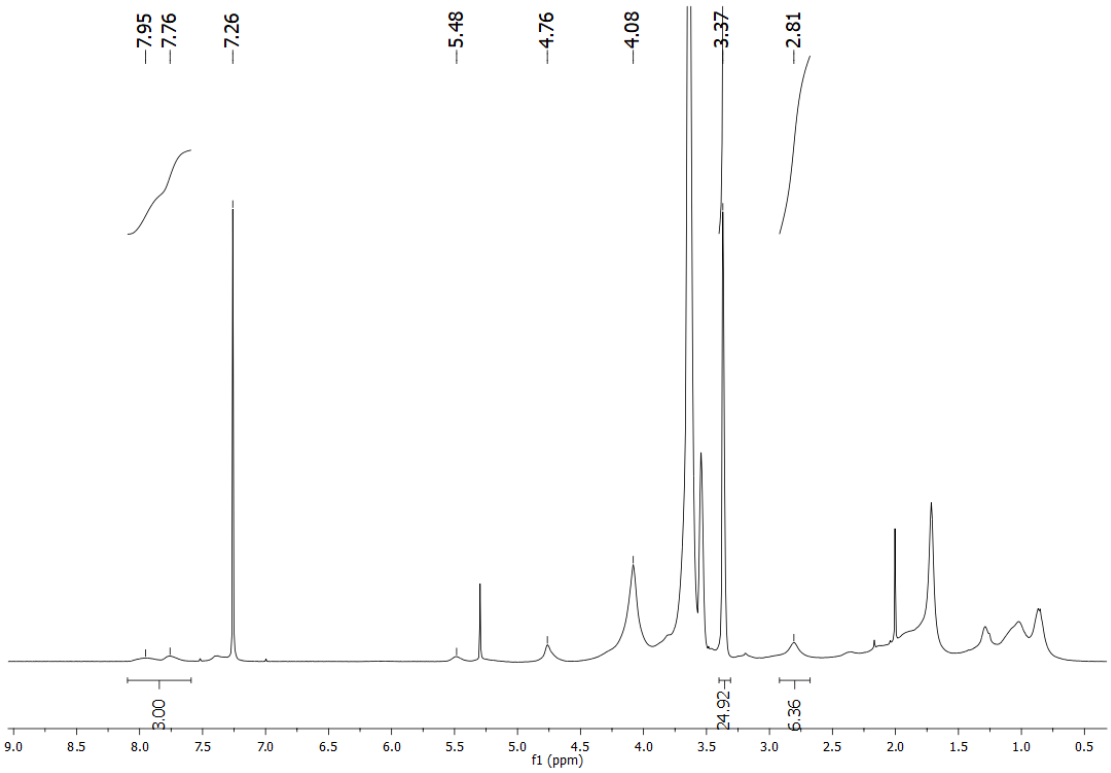

Figure A. 2. ^{1}H NMR spectrum of copolymer 5.

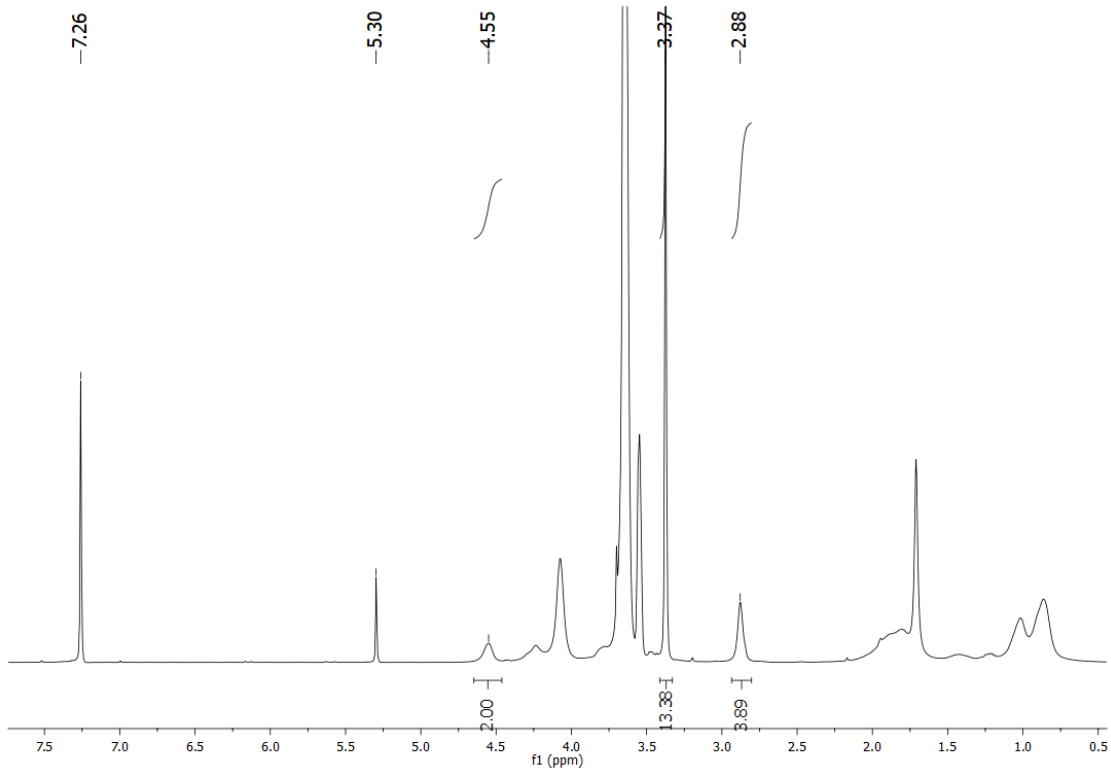

Figure A. 3. ^{1}H NMR spectrum of copolymer 7.

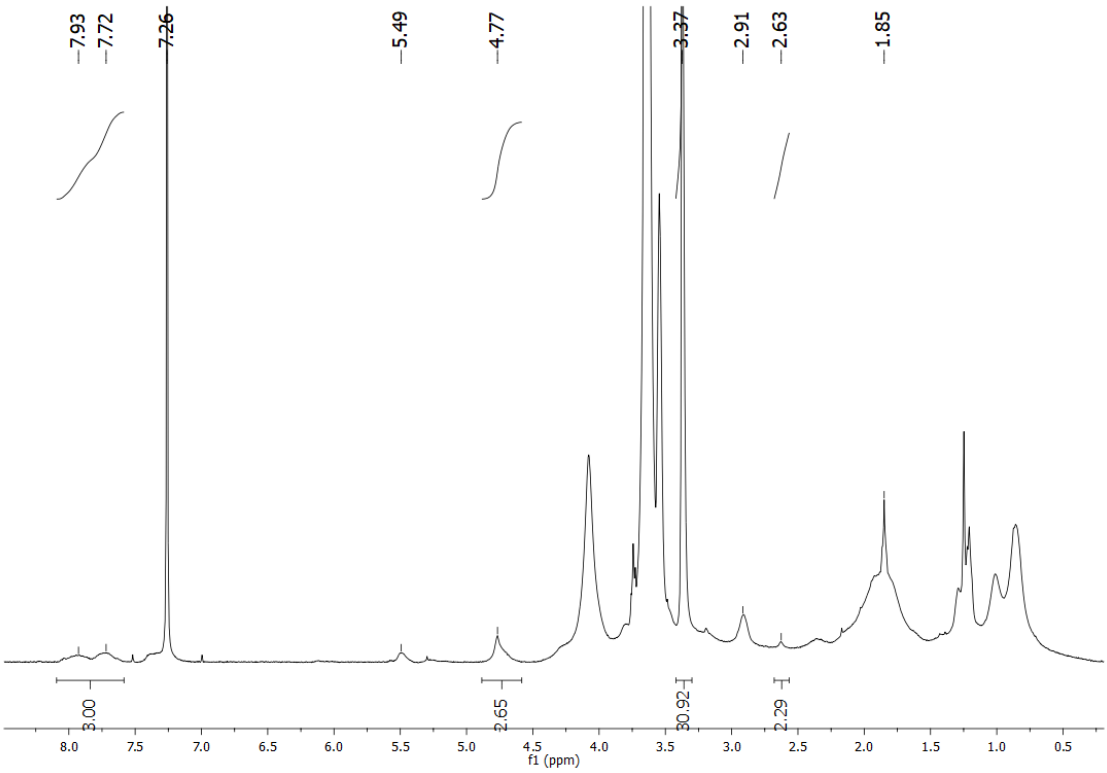

Figure A. 4. ^{1}H NMR spectrum of copolymer 8.

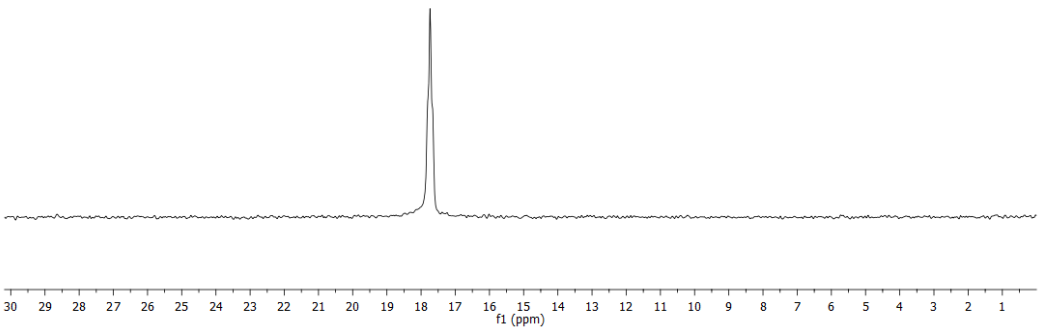

Figure A. 5. ^{31}P NMR spectrum of copolymer 8.

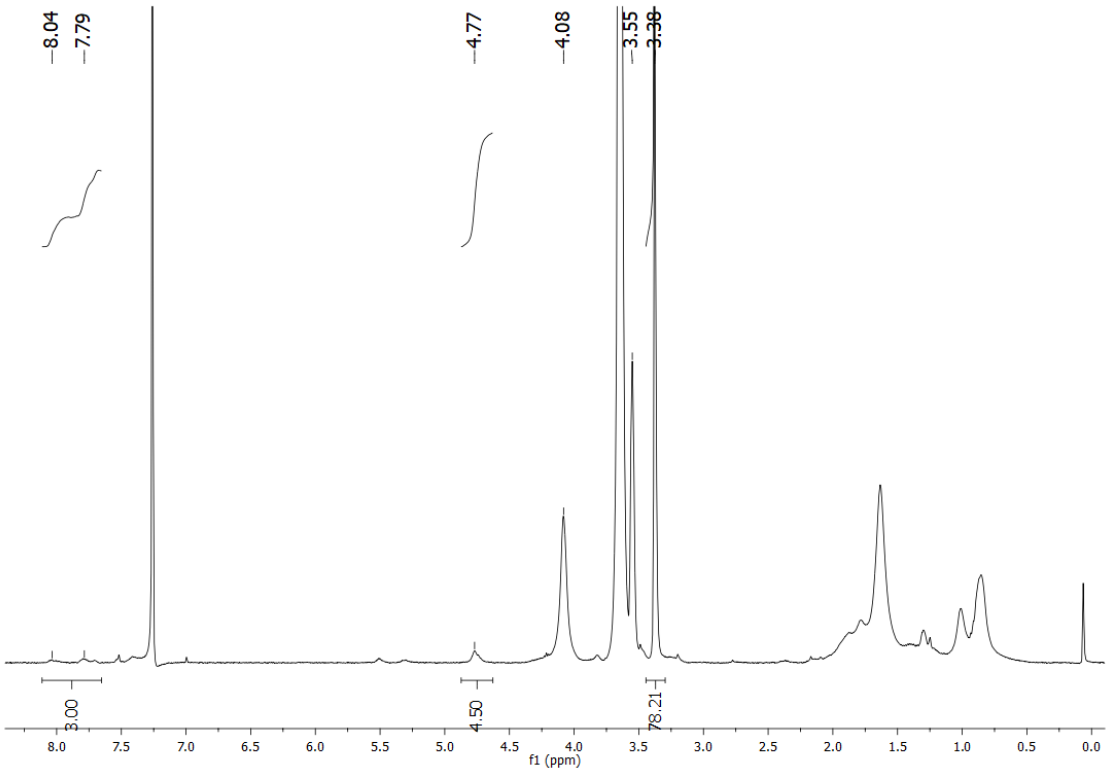

Figure A. 6. ^{1}H NMR spectrum of copolymer 9.

Table A.1. Fluorescence intensity of copolymer 8 in release medias.

pH / Day	5.4	7.4
1	4.715814	1.846689
2	1.962752	0.379303
3	1.343636	0.359534
4	1.259478	0.361984
5	0.704736	0.404894
6	0.647998	0.309897
7	0.766484	0.336199
8	0.721523	0.190417
9	0.585698	0.228469
10	0.653270	0.205008
11	0.286095	0.166441
12	0.618004	0.157947
14	0.389518	0.251923

Table A.2. Absorbance levels of supernatant of copolymer 8 and copolymer 9.

Time(min) / Copolymer	0	5	10	30	60
Copolymer 8	2.23234	1.34733	1.33068	1.28755	1.29422
Copolymer 9	2.38965	2.41162	2.40844	2.40058	2.35808

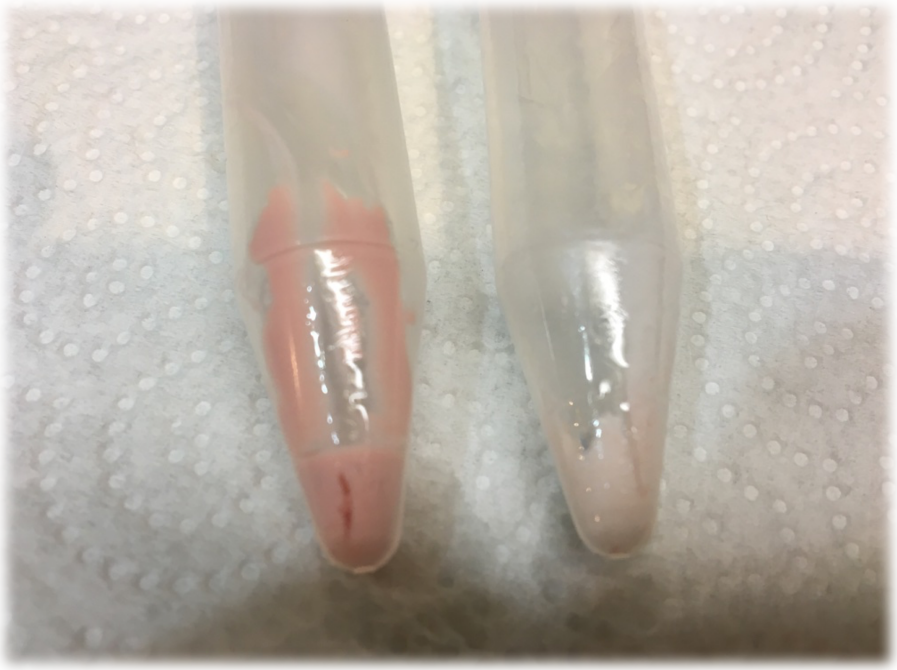

Figure A. 7. Color of precipitated HA crystals after incubation with copolymer 8 (left) and copolymer 9 (right).

APPENDIX B: PERMISSIONS FOR FIGURES

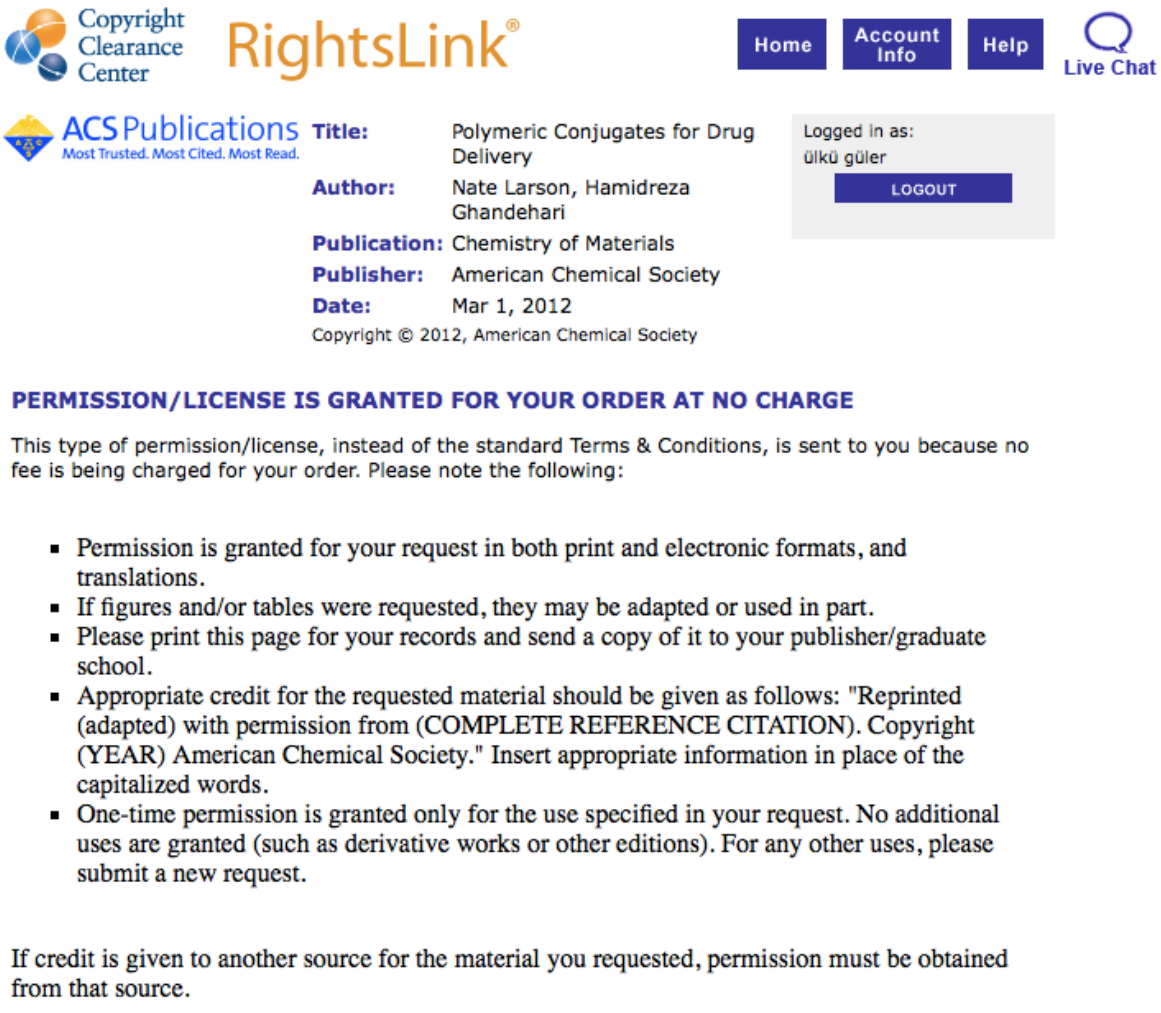

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Figure B. 1. Permission for figure 1.4

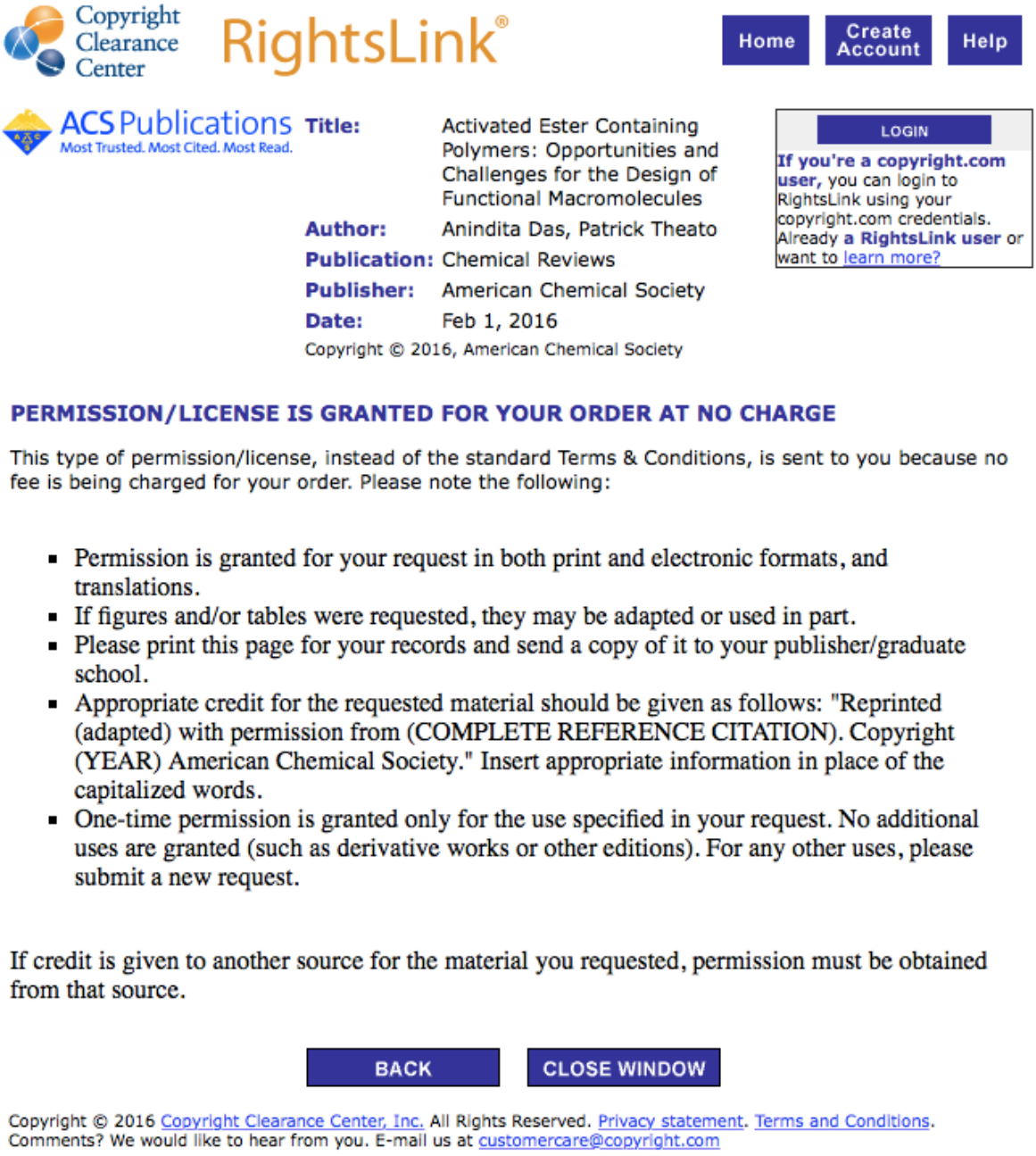

Figure B. 2. Permission for figure 1.5