INVESTIGATION OF ANTIBODY-DYE CONJUGATES

by

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To my superhero mother, Sebat Karagöz

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ABSTRACT

INVESTIGATION OF ANTIBODY-DYE CONJUGATES

Polymeric architectures are widely used in polymer therapeutics for both imaging and drug delivery applications. Design of these polymer drug conjugates consists of three parts: water soluble monomer, linker and the drug or imaging agent. Linkers are chosen according to the stimuli which is preferably overexpressed in the tumor environment. In addition to polymeric scaffolds, targeting agents can be introduced to these systems. As a targeted drug delivery agent, antibody drug conjugates have been used in the market recently for the treatment of cancer. Antibody ensures specific targeting of the cargo due to strong affinity to their receptors. However, these agents are limited by the number of drug molecules they can carry. To overcome this limitation, polymer is conjugated to antibody where the payload is carried by this polymer. As a model system, trastuzumab is conjugated to dye bearing polymers.

ÖZET

ANTİKOR BOYA KONJUGATLARININ İNCELENMESİ

Polimerik yapılar görüntüleme ve ilaç taşıma amacıyla yaygın olarak kullanılan terapatik ajanlardır. Polimer ilaç konjugatları üç parçadan oluşmaktadır: suda çözünebilen monomer, bağlayıcı ve ilaç veya görüntüleme ajanı. Bağlayıcılar, tümör çevresinde çok daha fazla bulunan bir uyarıcıya göre seçilir. Bu polimerik yapıya ek olarak, hedefleme ajanı da bu sistemlere eklenebilir. Hedeflenmiş ilaç taşıma ajanı olarak, antikor ilaç konjugaları son yıllarda piyasada kullanılmaya başlanmıştır. Antikorların reseptörlerine olan yüksek birleşme eğilimi sayesinde, spesifik bir hedefleme yapmak mümkün olmaktadır. Ancak bu antikorlar taşıyabilecekleri ilaç sayısı bakımından sınırlıdırlar. Bu sorunu aşabilmek için, istenilen yükü taşıyan polimer, antikora bağlanmıştır. Model sistem olarak trastuzumab antikoru boya taşıyan polimerler ile birleştirilmiştir.

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LIST OF ACRONYMS/ABBREVIATONS

ACN	Acetonitrile
ADC	Antibody Drug Conjugate
AIBN	Azobisisobutyronitrile
ALL	Acute Lymphoblastic Leukemia
$CDCl_3$	Deuterated Chloroform
CH_2Cl_2	Dichloromethane
CPADB	4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid
CTA	Chain Transfer Agent
Da	Dalton
DAR	Drug to Antibody Ratio
DCC	*N,N'*-Dicyclohexylcarbodiimide
DCU	*N*,*N'*-Dicyclohexylurea
DCM	Dichloromethane
DMAC	*N*,*N*-Dimethylacetamide
DMAP	4-Dimethylaminopyridine
DMF	*N*,*N*-Dimethylformamide
EPR	Enhanced Permeability and Retention
EtOAc	Ethyl Acetate
FDA	Food and Drug Administration
FMA	Fluorescein *O*-methacrylate
GFLG	Glycine-Phenylalanine-Leucine-Glycine
GPC	Gel Permeation Chromatography
GSH	Glutathione
HER2	Human Epidermal Growth Factor Receptor 2
HPMA	Hydroxypropyl Methacrylate
IV	Intravenous
LC-MS	Liquid Chromatography-Mass Spectrometer
mAb	Monoclonal Antibody
MED	Minimum Effective Dose
MeOH	Methanol

MMAF	Monomethyl auristatin F
MMAE	Monomethyl auristatin E
MTD	Maximum Tolerated Dose
NHS	N-Hydroxy Succinimide
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffer Saline
PDC	Polymer Drug Conjugates
PEG	Poly(ethylene glycol)
PEGMEMA	Poly(ethylene glycol) monomethyl ether methacrylate
PGA	Poly(glycolic acid)
PMMA	Poly(methyl methacrylate)
PT	Polymer Therapeutics
PVA	Poly(vinyl alcohol)
PVP	Poly(vinyl pyrrolidone)
R&D	Research Development
RAFT	Reversible Addition-Fragmentation Chain Transfer
RT	Room Temperature
SCID	Severe Combined Immunodeficiency Disease
SCPDB	4-Cyano-4-(phenylcarbonothioylthio) pentanoic succinimide ester
SEC	Size-Exclusion Chromatography
TLC	Thin Layer Chromatography
TW	Therapeutic Window
Tz	Trastuzumab
UV	Ultraviolet
Vis	Visible
WBC	White Blood Cell
WHO	World Health Organization

1. INTRODUCTION

1.1. Cancer and Treatment Methods

Cancer is a severe disease which affects many people around the world. World Health Organization has defined cancer as uncontrolled growth of abnormal cells (Figure 1.1) which can later infect neighboring cells and parts of the body, resulting in spreading to other organs [1].

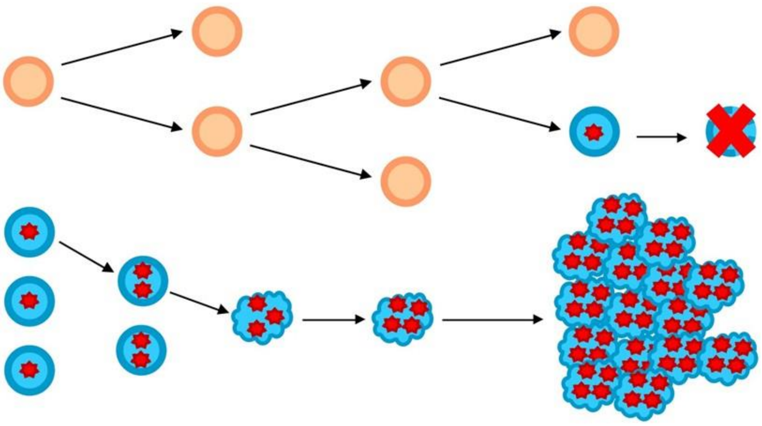


Figure 1.1. Cancer cell formation and growth

Cancer is resulted from genetic changes caused by physical factors like radiation and UV, by biological carcinogens such as some viruses (hepatitis, HIV) and bacteria (helicobacter pylori); by chemical carcinogens for example benzene and aromatic amines, and by environmental factors such as smoking and consuming overcooked red meat, fish [2-4]. Mentioned factors induce malfunctioning of some genes especially on oncogenes and tumor suppressor genes. These altered genes lead to neoplastic transformation which can be defined as basically conversion of healthy cells to cancer cells [4, 5]. Oncogenes are the permanently activated form of proto-oncogenes which play an important role in cellular growth and differentiation, encoding growth factors and synthesis of regulatory proteins in signal transduction [6]. When there is a need for certain proteins, growth factors or any mechanism mentioned above; proto-oncogenes are switched on until there are enough growth factors and proteins. In the case, where these proto-oncogenes are damaged and become permanently turned on (oncogenes), the cell keeps growing and may invade other cells boundaries. On the other hand, tumor suppressor genes work in detection and repair of DNA, cell cycle checkpoint responses and tumor angiogenesis [7]. If there is a damage on DNA, these genes try to repair it and if that is not possible, they tell cell to die which is known as apoptosis. When tumor suppressor genes are turned off, apoptosis process do not work properly and abnormal cells grows out of control resulting in cancer. In a healthy cell, tumor suppressor genes and proto-oncogenes work in balance. When this balance is disrupted, overexpression of growth factors and uncontrolled cell growths causes proliferation of cancerous cells. When they overcome their natural boundaries, they start to invade neighboring cells and tissues eventually spread through the lymphatic system in the body. This event is called as metastasis.

It is very hard to detect cancer at the beginning stages depending on various reasons such as not having a regular check-up, small tumor sizes which do not show up in scans, not having serious symptoms and early symptoms being similar to other diseases. Moreover, it is almost impossible to detect cancer when it is in situ meaning abnormal cells exist but do not invade the neighboring tissue. Also, depending on the cancer type and its mechanism of action, cancer may stay silent at the beginning for a long time and become invasive very fast.

Mentioned factors along with socioeconomic factors make it hard to detect cancer in early stages. When people diagnosed with cancer, mostly it is in later stages (2, 3 or 4). Once cancer has proceeded in advanced stages, it becomes a challenge to treat cancer. Moreover, that treatment may not be effective and eventually result in death. 43.8 million people have been diagnosed cancer in the last 5 years [8]. Among the noncommunicable diseases, cancer is the second leading cause of death around the world [9]. The number of people who has died because of cancer has been increasing every year. In 2016, 9 million people died because of cancer [9]. In 2018, this number has been raised to 9.6 million where 18 million new cases have been reported in 2018 all around the world [8]. When considering these numbers, importance of studying cancer is understood better.

Among these rates, breast cancer is placed in second line considering the number of new cases (2 million, 11.6%) and in 5th line considering the number of deaths (626 thousand, 6.6%) in 2018 [10]. Among the causes of breast cancer, the gene called Human Epidermal growth factor Receptor 2 (HER2) outstands. HER2 gene plays a role in cell cycle like cell growth, division and cell repair [11]. In a healthy cell, two copies of HER2 exists. Around 20% of breast cancer is raised from over expression of HER2 gene and called HER2 positive breast cancer [12-14]. HER2 positive breast cancer have more aggressive progress profile than others. In general, rapid tumor proliferation causes metastasis and lowers survival rates. Moreover, reoccurrence of cancer is also highly possible.

Today’s treatment methods of cancer involve radiotherapy, surgery and chemotherapy. Radiotherapy enables some success but it is not suitable for all cancer types. It depends on several factors such as type of cancer, location of the tumor in the body, size of tumor, patient’s medical conditions and age. Moreover, for some patients it might not be the only treatment they need, radiotherapy may be applied to shrink tumor and for the further cure, they still may need chemotherapy or surgery. Additionally, it is very hard to point exact cancerous tissue and border between healthy tissues.

Surgery is another option to treat cancer; however, it is also not applicable for every case. It gives better results if the tumor is solid and contained in one area. If cancer has spread to other organs and bones, surgery is out of option. Moreover, it might not be possible to remove tumor as a whole if taking it causing a severe damage on organ. This means there are still left over tumor in the body. In the cases which are suitable for surgery, there is still number of risks. These includes, spreading cancerous cells during operation, during blood circulation that cell can settle to another place and cause cancer reoccurrence. Even if this is not the case, patients still receive follow up treatments, especially chemotherapy. Other risks are related to surgery itself like risk of infection, bleeding, etc.

Regular chemotherapy methods require use of agents specially drugs with high toxicity. Unfortunately, these drugs also have severe drawbacks such as rapid metabolism and clearance from body, low solubility in the blood, short half-life during blood circulation and smaller therapeutic window which can be defined as the gap between maximum tolerated dose (MTD) and minimum effective dose (MED) of given drug (Figure 1.2).

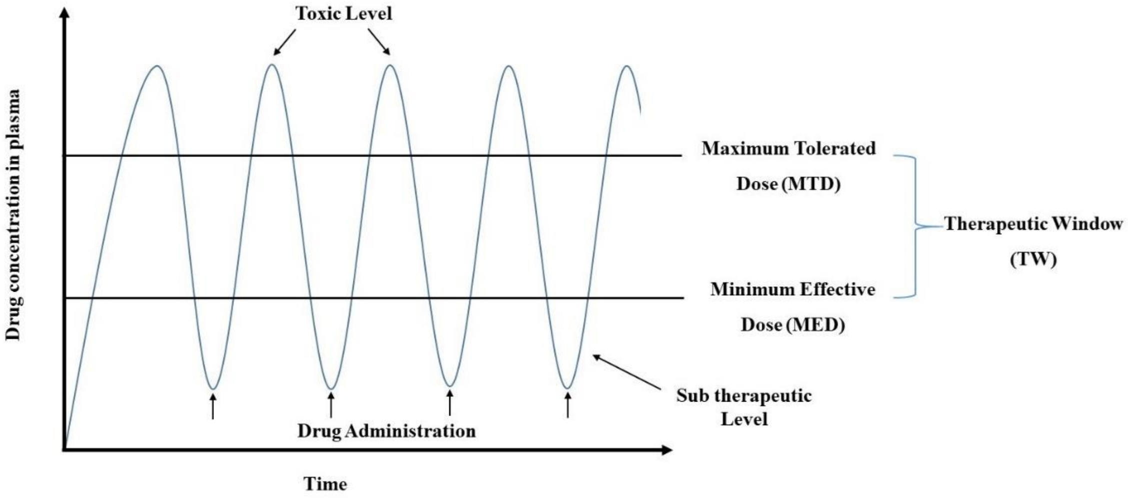


Figure 1.2. Therapeutic window representation in conventional chemotherapy

Narrow therapeutic window, limits the effectiveness of the drug and the course of treatment. In order to have successful result from the therapy, the given drug concentration must be above the MED. Rapid metabolism and clearance causes sudden decrease in drug concentration in the blood and if drug has narrow therapeutic window, that concentration may go below the MED very easily. To keep the drug concentration in blood plasma above MED, frequent drug administration is needed. This may cause two major drawbacks. Sudden increase in concentration causes crossing over the MTD and side effects are stated to experience. Above the MTD, drug becomes more toxic than usual due to increased penetration. For this reason, most patients are given a rest time to recover from these side effects, allowing enough time to recover the immune system and produce more white blood cells. However, these frequent administrations and breaks from therapy causes tumor to develop a resistance against the drug. There are already limited options in chemotherapy. Once body starts to develop resistance, success rates of other chemotherapy agents are also lower.

Even if TW is relatively in acceptable ranges, short half-life of the drug also causes decrease in concentration of the drug in the blood in a short period. This also requires frequent drug administration. Especially in chemotherapy frequent dosage is not wanted not only because the risk of developing resistance against the agent but also experiencing heavier side effects.

Since chemotherapy agents are designed to kill fast growing and dividing cells, along with being lack of targeting; they are toxic to not only cancerous cells but also all fast growing cells in the body such as hair follicles, sperm and white blood cells (WBC). This is the reason why people experience hair loss, nausea etc. Attacking WBC means that attacking patients own immune system, and patient becomes vulnerable to any disease. Even if they get simple flue, they cannot fight it and cannot receive any other medication.

Another drawback of chemotherapy drugs is low solubility in the blood circulation. These drugs are mostly hydrophobic, in order to have pharmacokinetic activity, they must be internalized to cell which has aqueous media. Being hydrophobic do not ease their internalization. In order to solubilize these drugs, they are administered with auxiliaries. Unfortunately, these auxiliaries mostly more toxic than drug itself. These leads to experience more side effects.

All mentioned factors cause a decrease in bioavailability of the drugs directly or indirectly. It is possible to think bioavailability as how much of the given drug reaches the site of action. For example, bioavailability of a drug is 100% if it is administered intravenous (IV). Until it reaches its destination, drug travels through the blood stream and sometimes they start to become active or decompose before they reaching site of action. Moreover, to penetrate tissue they must get through some barriers. Finally, the drug concentration, reaching the destination and still being able to fulfill their duty, is less than 100%. If the drug has low solubility, it gets metabolized before reaching the destination, gets cleared very fast or has a short half-life, its bioavailability lowers dramatically. In this case, it is hard to keep drug concentration above MED and patients need frequent drug administration. This also leads experiencing more side effects and increased chance to developing drug resistance.

Taking into consideration all mentioned factors above, most people die not because of cancer itself but because of these severe side effects of chemotherapy agents. Even if the chemotherapy is the most common used therapy against cancer, it is still not the best option. In order to address some of these drawbacks, scientists start to develop some modifications on these chemotherapy agents. One of the most promising area is polymer therapeutics.

1.2. Polymer Therapeutics

The term “polymer therapeutics (PT)” is introduced by Ruth Duncan and it implies for all polymer structures applied in therapeutic area such as contact lenses, prostheses, implants, patches, medical devices like stents, dental applications and pharmaceutical excipients [15]. In pharmaceutical area, scientists aim to use polymer therapeutics as a drug carries designed to deliver the drug to site of infection without losing its therapeutic effect and improving its therapeutic index. Historical progress of PT, starts with the discovery of liposomes in 1965 followed by dendrimers in 1978 and PEGylated liposomes in 1980s [16]. In this scope, polymeric drugs, polymer-drug conjugates, polymer-protein conjugates, polymeric micelles where drug is covalently bound have been developed [17-24].

Since that time, there has been several number of PT agents are entered clinical trials and many of them approved by FDA. The introduction of polymers to pharmaceutical area as a drug delivery system is done by Ringsdorf in 1975 and become biologically applicable by the progress of Duncan and Kopecek in 1984.

Design of a PT system mostly aims to address the problems in regular chemotherapy such as being lack of targeting, low solubility and to increase therapeutic efficiency. For this reason, they are mostly preferably biodegradable and biocompatible polymeric backbone where the pharmaceutical agent is conjugated by covalent linkers or physically entrapped by the molecular interactions.

Developments in both organic and polymer chemistry, enable scientist to synthesize and design various polymer scaffolds which can be functionalized with linkers, imaging and/or targeting moieties, solubilizing agents and polymers having more controlled molecular weights with tunable physical properties [25, 26]. In the scope of PT, this project is focus on polymer-drug conjugates.

1.2.1. Polymer Drug Conjugates

Polymer drug conjugates (PDC) are introduced by Ruth Duncan and shortly after this development, first PDC was entered clinical trial [27, 28]. Constructive components of the polymer drug conjugates can be classified in three parts: monomer, linker and therapeutic agent. In addition to these, sometimes targeting moiety can be attached (Figure 1.3).

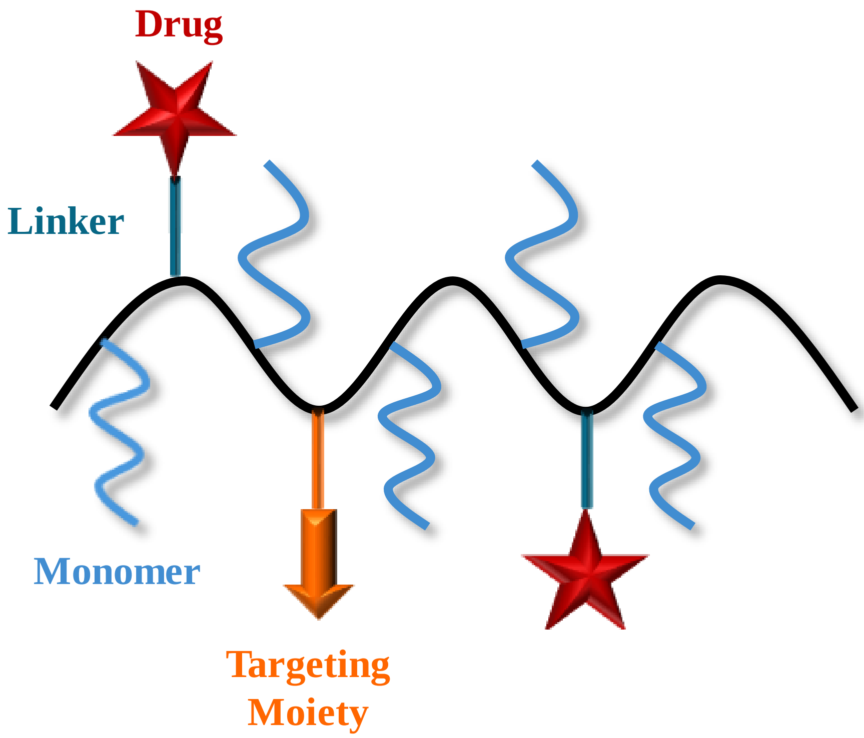
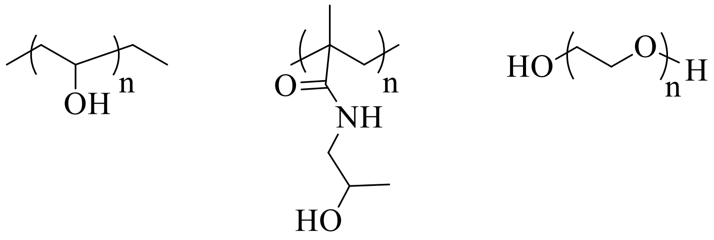


Figure 1.3. Representation of polymer drug conjugates

There are various options for the monomer; however, in order to solve the solubility problem of chemotherapy drugs, generally hydrophilic monomers are chosen. Some examples for these monomers are poly(vinyl alcohol) (PVA), poly(glycolic acid) (PGA), poly(maleic acid), poly(vinyl pyrrolidone) (PVP), poly(hydroxylpropyl methacrylate) (HPMA) and poly(ethylene glycol) (PEG) (Figure 1.4). From these, biocompatible ones such as HPMA and PEG are mostly used for PDC design.



PVA **pHPMA** **PEG**

Figure 1.4 Examples of water soluble monomers

PEGylation is the term for PDC's where drug is conjugated to poly(ethylene glycol). Choose of PEG as a water soluble monomer has additional features other than being biocompatible; like anti-immunogenicity, anti-biofouling and high flexibility. It protects the bioactive moiety from the immune system and early degradation in the blood stream by decreasing the interactions with enzymes. This increases the bioavailability of the drug which is one of the aims of PDC. When it comes to especially in protein conjugates, PEGylation becomes a very attractive choice. It increases the volume of the conjugate in the water ensuring that longer circulation time hence longer half-life and slower renal clearance. There are several PEGylated drug and/or protein conjugates in the literature. Some of them even succeed to go in the market for the treatment of some diseases (Adagen® for the treatment of SCID associated with a deficiency of adenosine deaminase, 1993) as well as cancer (Oncaspar® for the pediatric and adult patients with ALL) [29, 30]. Other advantage of PEGylation can be their ability to act as a spacer during polymerization. If the conjugated molecule is large, there is a high possibility to have steric hindrance between these molecules. This may lower the number of conjugated molecule. Playing role as a spacer can decrease this hindrance hence increase the number of conjugations.

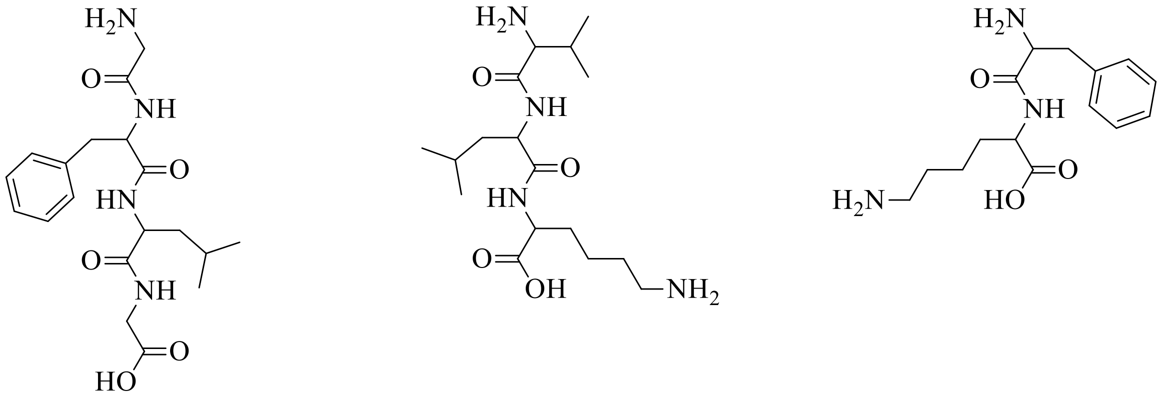
Another component of polymer-drug conjugates is linker. Linkers also may serve as spacer in order to decrease steric hindrance hence to increase the number of conjugations along with delivery of the cargo and/or serve as on command release of the therapeutic agent where the trigger can be internal stimuli (enzymes, pH) or external stimuli (temperature, light).

Linker must protect the bioactive compound from premature release in the blood circulation; however, it must enable the release of the drug when target area is reached. If the linker is too stable, it will not release the drug when the action is needed. This leads to the drug concentration being below the MED and therapeutic failure. If it is not stable enough, drug is released early and toxic effects are seen in unwanted areas. This also causes low bioavailability, higher side effect profile hence poor pharmacokinetics. For this reason, linker design should be optimized such a way that it is stable enough to carry and protect the payload from external circumstances during its transportation, and yet ready to release the payload when action is necessary. Other requirements which needs to be satisfied by the linker include biocompatibility and/or biodegradability, mild synthesis circumstances to keep the activity of the drugs, and relatively faster cleavage in the presence of stimuli [31].

Internal stimuli responsive linkers can be classified as pH responsive, redox responsive and enzyme responsive linkers. It is known that tumor tissue has a slightly acidic media (pH around 5.5) caused by the increased concentration of lactic acid which results from further reduction of pyruvate produced in glycolysis step by rapidly growing and proliferating cancerous cells. In order to take advantage from this pH difference, linkers that are broken in acidic media such as carbamate esters, imines, carbonate esters and carbonates are used for PDC design.

In some tumor tissues like ovarian, lung and breast tumors, GSH concentration which serves as a reducing agent, is elevated compared to healthy tissues [31]. Moreover, intracellular concentration is much higher than extracellular concentration. This knowledge leads people to design redox responsive linkers such as disulfide linker.

Drug delivery systems which designed to be enzyme responsive, have additional features such as catalytic efficacy, sensitivity and selectivity towards their substrate and biorecognition. Enzymes have high affinity to their substrates thus showing higher efficiency. In drug delivery systems, scientists take advantage of the abundance of special enzymes in the tumor site due to their selectiveness towards their substrates. Moreover, enzymes have lower concentration in blood circulation compared to tumor tissue. Based on these facts, enzyme responsive linkers are degraded primarily in tumor site. Another advantage of enzyme sensitive linkers is their biodegradability since they are designed to be enzyme’s substrate. This ensures there will be no toxic residues after they have undergone lysosomal pathway resulting in improved pharmacokinetic profile. Based on these information, many cleavable peptide linkers have been designed such as dipeptides and tetra-peptides as response to different enzymes like matrix metalloproteases, cathepsins and plasmin. For example, GFLG linker as response to Cathepsin B and Val-Leu-Lys tripeptide that can be cleaved by plasmin enzyme (Figure 1.5) [31].



GFLG **Val-Leu-Lys** **Phe-Lys**

Figure 1.5. Examples of enzymatically cleavable linkers

Peptidase enzymes break down proteins by hydrolyzing the peptide linkers [31]. Cancerous cells have shown increased proteolytic activity which helps them to digest extracellular matrix (ECM). When ECM is destroyed, cells lose contact with their environment which is a perfect situation for infiltration of cancerous cells [32]. Cathepsins are one of the largest classes fall into peptidases. It has been proved that cathepsins are overexperred in various malignant cancer types such as breast, lung and colorectal cancers. [31]. It is shown that Cathepsin B overexpression is directly related to inflammatory breast cancer invasion [33]. Based on these information, linker was chosen as GFLG in this work.

Last structural component of PDC is chemotherapy agent. Choice of drug is based on targeted diseases. In this work, docetaxel is chosen since it is already used for the treatment of breast cancer.

In summary, there are various option for the design of PDC and the choice depends on the aim to be served. However, their general aim to address some of the problems in regular chemotherapy such as high toxicity, narrow therapeutic window, frequent drug administration and high side effect profile. Designing PDC, can increase the therepautic window, decrease number of drug administration by ensuring sustained release due to stimuli responsive linkers, increase the drug concentration in tumor area hence increase the bioavailability of drug precursor (Figure 1.6).

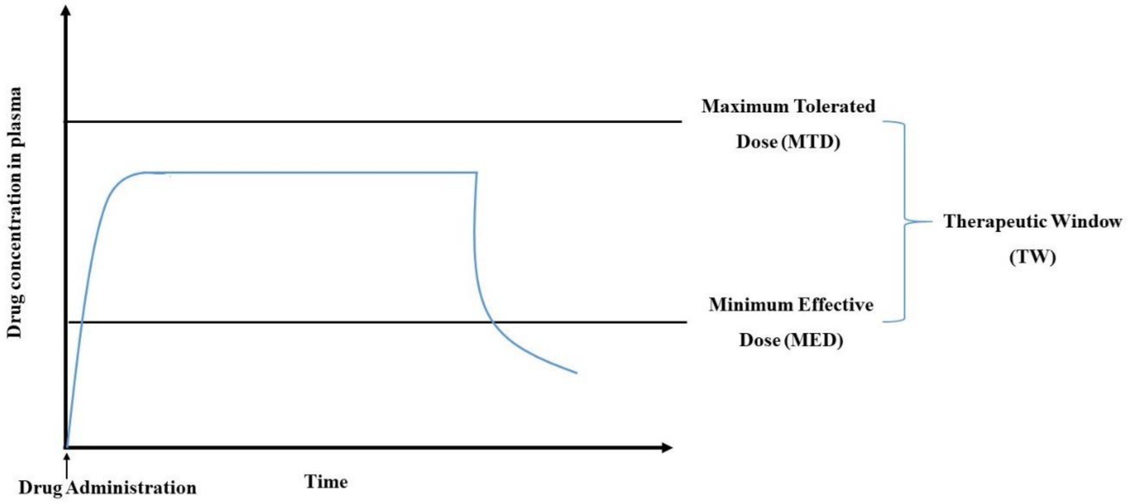


Figure 1.6. Representation of therapeutic window in polymer drug conjugates

1.2.2. Passive Targeting

It is well known that cells around the blood vessel in tumorous area are not well organized; in other words, there are larger gaps between the cells. On contrasts, cells around the blood vessel in healthy tissue are well defined and they have very small gaps. Small molecules, like regular chemotherapy agents, circulating in blood vessels can pass through the cells which are in both healthy area and cancerous area. On the other hand, larger molecules do not have ability to pass through the cells around healthy tissue whereas they can still go into the tumors area between the gaps. This gives tumor tissue to enhanced permeability ability. Another difference between healthy and tumor tissue is having or lacking of lymphatic drainage system. Healthy tissues have this system, so they are able to get rid of their metabolic waste through lymphatic drainage. On the other hand, tumor tissue does not have this system. Whatever comes in tumorous area stays in there. This gives them to retention ability. Overall explained phenomena is called “Enhanced Permeation and Retention” (EPR) effect and found by Matsumura and Maeda in 1986 [34].

Using polymer drug conjugates as a carrier, have an additional advantage other than mentioned above section. Such a design enables their accumulation in tumor tissue rather than healthy ones by taking advantage of EPR effect. In other words, design of polymer drug conjugates ensures passive targeting due to their larger sizes (Figure 1.7).

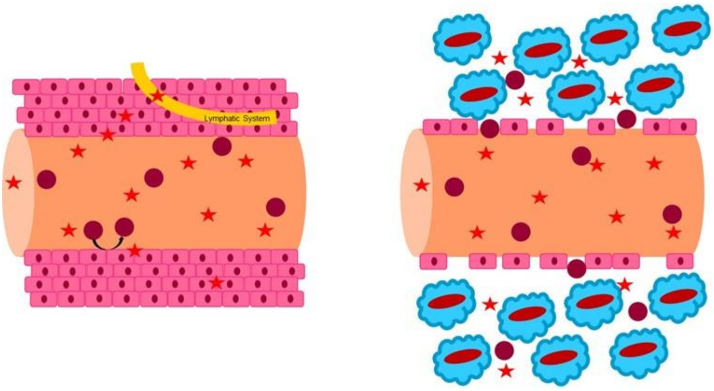


Figure 1.7. EPR Effect

Accumulation of PDC’s in tumor area provides increased local drug concentration hence increased bioavailability. Since therapeutic agent is expected to be inactive when they are conjugated to polymer backbone and is expected to release the cargo after PDC has reached desired area, overall toxicity against untargeted area is decreased. As a result, therapeutic index of chemotherapy agents is improved.

1.2.3. Active Targeting

The concept of active targeting, which can be explained as the delivery of cytotoxic drug to tumor via targeting agent in a selective way, was proposed by Paul Ehrlich in 1900’s [35, 36]. Based on this concept, polymer drug conjugates can be delivered to tumor site via active targeting. This can be achieved via conjugation of targeting moiety such as folic acid and RGD tripeptide, to polymer backbone. These ligands target certain receptors on cancerous cells. Hence, they are internalized via enzyme – receptor mediated internalization pathway and release the drug after lysosomal degradation.

Another way to target tumor tissue via active targeting is to use antibodies as a targeting moiety and conjugate the toxic agent to them. This concept is called as Antibody Drug Conjugate (ADC) which will be explained in section 1.3.

To sum up, all these constructs falling into polymer therapeutics mainly aims to improve pharmacokinetic profile and therapeutic efficiency by increasing the therapeutic window (Figure 1.6). By this way, minimum effective dose can be lowered and/or maximum tolerated dose can be increased. Since they are designed to be localized in tumor environment, bioavailability of the drug will be increased hence minimum effective dose can be decreased. On the other hand, polymers protect the drug from early release hence lower the toxic effects on healthy tissues. This increases the maximum tolerated dose. Moreover, on demand release of drug causes the sustained concentration in blood plasma hence, lower the number of drug administration. This can help to prevent improving drug resistance.

1.3. Antibody Drug Conjugates

The active targeting concept defined by Ehrlich, was applied to antibody where it plays a role as targeting agent in 1950’s. In general, antibodies are able to target the receptors and disable the proliferation of cancerous cells but they do not kill the cancer cells. In order to eliminate this deficiency, conjugation of killing agent was required. This is where the great development has occurred by the work of Georges Mathé in 1958. He conjugated the drug called methotrexate (MTX) to leukemia cell targeting antibody [37]. This work brings the concept of Antibody Drug Conjugates (ADC) and representative design was shown in Figure 1.8. Since then, design of ADC's has been changed from polyclonal antibodies linked to an agent both non-covalently and covalently to monoclonal antibodies covalently linked to drug molecules.

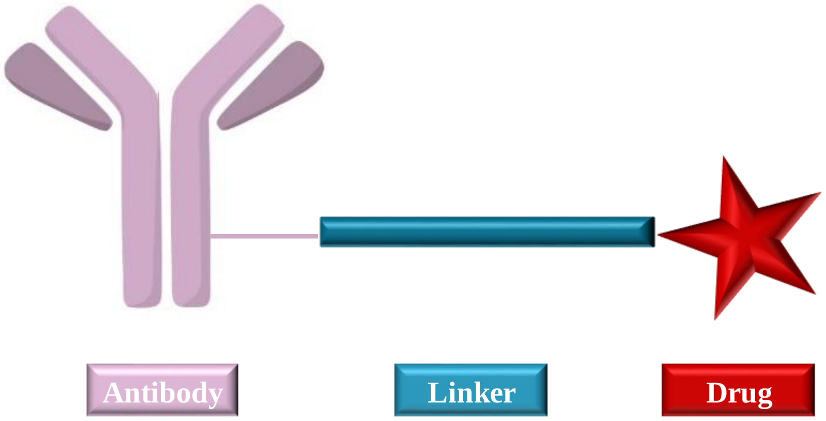


Figure 1.8. Representation of Antibody Drug Conjugates

Structural components of ADC include monoclonal antibodies (mAb), cytotoxic payload and linker between the drug and mAb. Antibody choice depends on the receptor that has been intended to be targeted. There are several mAbs and some of them were used as a treatment agent by approval of FDA. For example, Herceptin is a drug that is basically an antibody named Trastuzumab, used for the treatment of human epidermal growth factor receptor 2 (HER2) positive metastatic breast cancer. Trastuzumab (Tz) targets the HER2 receptors on the cancerous cell membranes and internalized via endocytosis. Moreover, it is shown that Tz inhibits angiogenesis *in vivo* [38].

As for the linkers, concerns about PDC design are valid for ADC design to. Ideal linker has to balance the needs between the efficient cleavage after delivery of warhead into the targeted cell and enhanced stability in the blood circulation during several days. To this end, cleavable, reducible, non-cleavable (or non-reducible) and polar linkers have been designed.

Cleavable linkers include enzyme responsive ones like valine-citrulline (Val-Cit) in ADC called brentixumab vedotin marketed as Adcetris® and pH responsive ones such as hydrazone.

As a reducible linker, disulfide bridges were chosen in several ADC’s. Non-cleavable linkers include maleimidocaproic acid tied to Monomethyl auristatin F (MMAF) and thioether linker succinimidyl 4-(N maleimidomethyl) cyclohexane-1-carboxylate linked to cytotoxic agent DM1 (SMCC-DM1) as used in the ADC called trastuzumab emtansine marketed as Kadcyla®. N-hydroxysuccinimidyl 4-(2 pyridyldithio)-2 sulfobutanoate (sulfo-SPDB) and mal-PEG4-N-hydroxysuccinimide are examples of polar linkers which are used in ADC design [39].

Antibody drug conjugates (ADC) has been trending due to their several advantages and the most important one is being able to target the specific receptor on cancerous cells. By this way, activity of highly toxic drug molecules is decreased against healthy cells. However, there are some limitations in both synthetic pathway and number of conjugated drugs. Drug to antibody ratio (DAR) cannot be raised too much without compromising the binding efficiency of that antibody, even if there are available conjugation sites. Adcetris® has the highest DAR value being 4 and Kadcyla® has 3.5 where both are FDA approved ADC’s on the market.

Another reason for not increasing DAR value is the extereme cytotoxicity of the drugs that are used. In the case of Adcetris, antimitotic drug derivative MMAE is used as a cytotoxic agent and this drug is too toxic to be used as an anticancer drug without conjugation. This situation is similar for Kadcyla as well. Maytansine derivative DM1 agent is also failed to be a chemotherapy agent alone in clinical trials due to its high systematic toxicity. Considering this much of high toxicity, increased DAR values such as 8 showed unwanted side effects. As a result, DAR around 4 is optimum value for both therapeutic effect and side effect profile [39-41].

1.3.1. ADC’s: Historical Perspective, Clinical Trials and Market Products

ADC concept has been sparkly improved after the development of mouse mAbs via hybridoma technology by Kohler and Milstein in 1975 [42]. Shortly after this development, first human clinical trials were started with ADC against advanced metastatic adenocarcinomas [43]. Figure 1.9 shows timeline of ADC’s [37].

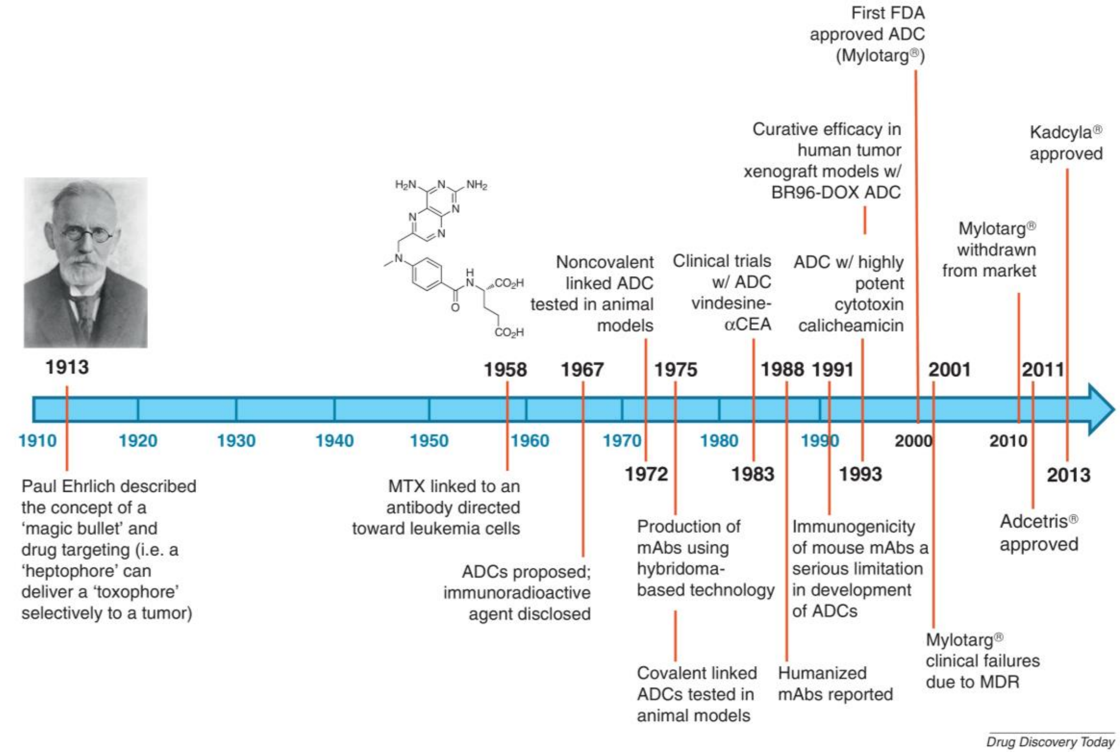


Figure 1.9. Historical progress on ADC [37]

ADC’s that are in the market and some examples of ADC’s which are in clinical trial shown in Table 1.1.

Table 1.1. Examples of ADC’s on the market and in clinical trial

Agent	**Status**	**Toxic Agent**	**Indication**	**Sponsor (Licensee)**
Brentixumab vedotin, Adcetris®	In market since 2011	MMAE	HL and ALCL	Seattle Genetics (Millennium)
Trastuzumab emtansine, Kadcyla®	In market since 2013	DM1	HER2+ metastatic breast cancer	Roche-Genentech (ImmunoGen)
Gemtuzumab ozogamicin, Mylotarg®	Withdrawn from market in 2011, in market since 2017	Calicheamicin	AML	Pfizer (UCB)
Inotuzumab ozogamicin, Besponsa®	In market since 2017	Calicheamicin	ALL and NHL	Pfizer-Celltech (UCB)
Glembatumumab vedotin	Phase II	MMAE	Advanced breast cancer, melanoma	Celldex (Seattle Genetics)
Milatuzumab-DOX	Phase I/II	Doxorubicin	MM	Immunomedics
SAR-566658	Phase I	DM4	Solid Tumors	Sanofi (ImmunoGen)

1.3.2. Strategies for Obtaining Antibody Drug Conjugates

Chemical conjugation of drug molecule through a linker to an antibody was done via amino acid residues on the side chains on the mAb. These amino acids are commonly cysteine and lysine.

Considering the lysine amino acid, there are several available chemical reaction options based on amine group on the side chain of lysine. One approach is direct conjugation of amine to NHS ester bearing drug-linker to form stable amide bond (Figure 1.10). This approach is used in Mylotarg® [44]. Another method includes two-step process. First, amine groups on lysine residues are modified to reactive species like maleimide or thiol. In the case where amine is modified to maleimide, Michael addition becomes favorable with variety of functional group such as thiol. This approach is used in Kadcyla®. Maleimide chemistry is widely used for conjugations because the ease of reaction in physiological conditions. However, formed thiosuccinimide bond can be broken slowly in blood circulation. Majority of ADC's having this chemistry and going in clinical trials experienced measurable drug loss during circulation hence showed poor pharmacokinetic profile [39].

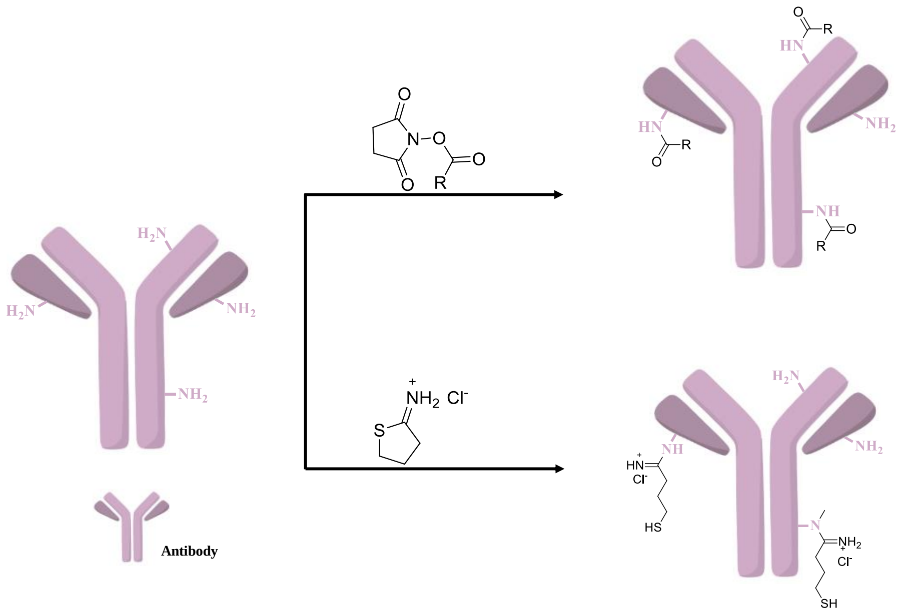


Figure 1.10. Conjugation chemistry based on lysine amino acid

Considering two-step process, first step can be modification of amine group to thiol. After this modification, Michael addition reaction yielding thioether bond, halo-carbonyl alkylations and disulfide formation become available options for reactions [45].

If a conjugation is planned to be done on cysteine residues, reduction of disulfide bridges between the heavy chains of mAb is required (Figure 1.11) [45]. Ones it is reduced, free thiol groups become available for conjugation via different reactions mentioned above [46-48]. Partial reduction of disulfide bridges on mAb and conjugating via thiol maleimide chemistry is applied for Adcetris® [37, 49]. However, mild reduction of these disulfide bonds require great control and attention. If both bridges between the heavy chains are reduced, mAb breaks down into two each carrying one heavy and one light chain. Another danger is to break the bridges between heavy and light chain, which results in again destruction of mAb.

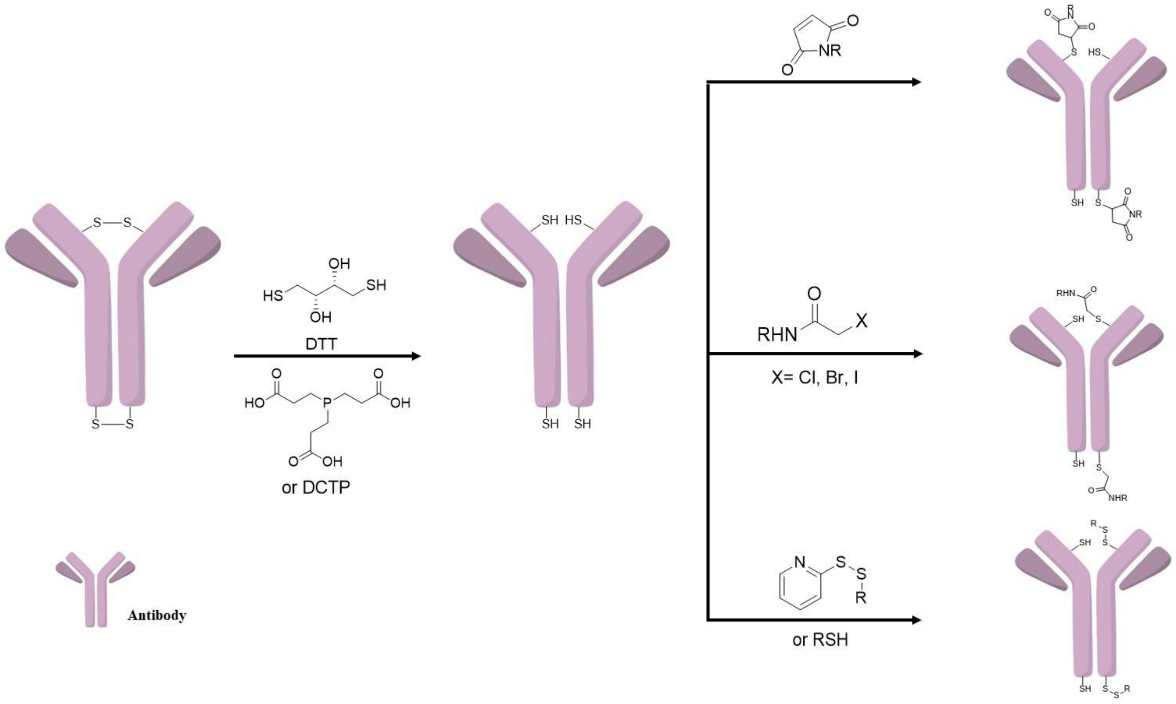


Figure 1.11. Conjugation chemistry based on cysteine amino acid

1.4. Chemistry Behind Drug Carrier Systems

1.4.1. Polymerization Techniques for Polymer Drug Conjugates

Advancements in polymer chemistry offers broad options to design polymers with different architecture, high uniformity, controlled molecular weight and conjugation of different monomers/reactive groups of interest to polymer backbone. There are several controlled chain growth dependent polymerization strategies such as atom transfer radical polymerization (ATRP), reversible addition−fragmentation chain-transfer polymerization (RAFT), nitroxide-mediated radical polymerization (NMP), ring-opening metathesis polymerization (ROMP) and Ring-opening polymerization (ROP). The polymerization technique used in this thesis is Reversible Addition-Fragmentation Chain Transfer (RAFT) polymerization.

RAFT polymerization is a method of living controlled radical polymerization. Which uses a radical initiator and chain transfer agent (CTA) for the generation of polymers with desired molecular weights and low polydispersity index values. It is mostly applied for the polymerization of (meth)acrylates, (meth)acrylamides styrenes and its derivatives, vinyl group conjugated to a double bond and nitriles like acrylonitrile. [50]. Polymers with a variety of end groups can be designed via RAFT polymerization such as, end group allowing the coupling of bioactive molecules, depending on the design of the RAFT agent (CTA) itself [51]. The mechanism of RAFT polymerization is shown in Figure 1.12 [50].

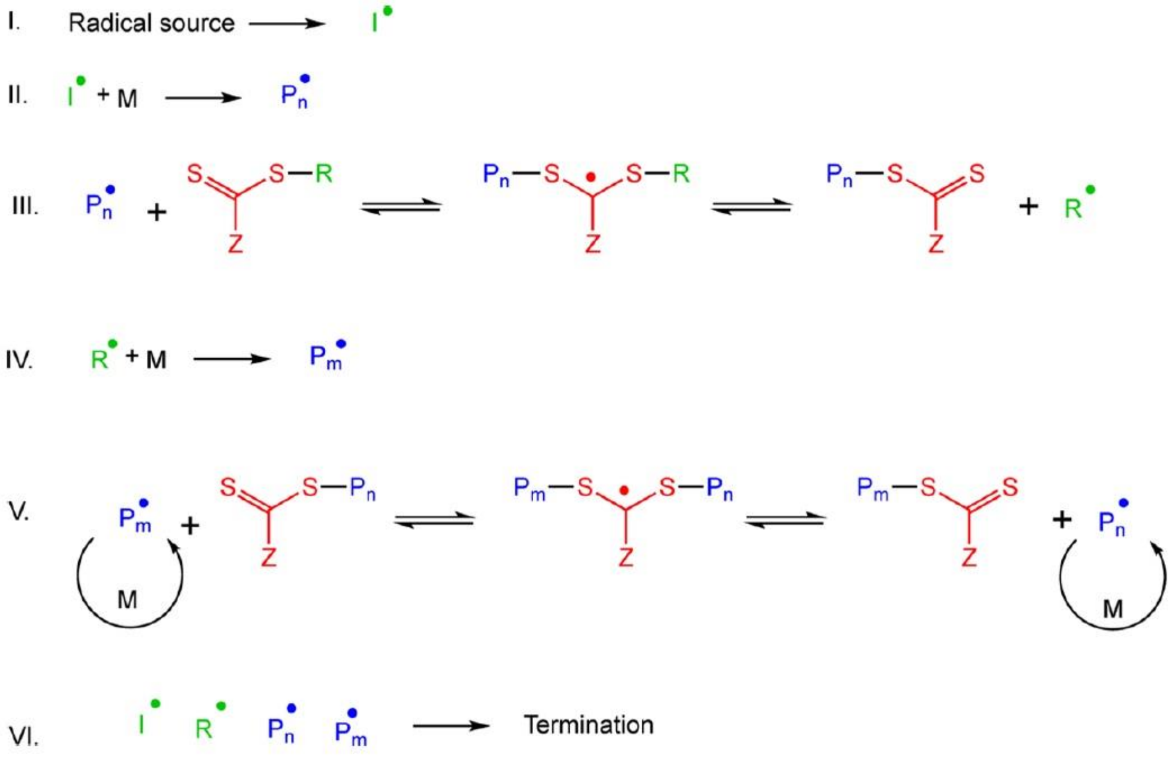


Figure 1.12. Mechanism of RAFT Polymerization [50]

The mechanism of RAFT polymerization is based on an equilibrium between dormant and active chains, achieved via degenerative transfer. In this system, there is no change in the overall number of radicals during the activation−deactivation process. First step is the activation step where the radical species were obtained. As a next step, obtained radical attack the monomer and polymerization starts. Obtained macromolecules which carry the radicals, were added to the RAFT agent (chain transfer agent, CTA) in order to enter equilibrium between active and dormant species as in steps III and V. Basis of the RAFT mechanism is formed by the chain transfers steps that form the basis of the RAFT mechanism. Chains were degenerated between the dormant chains of CTA which are typically thiocarbonylthio group, Z−C(=S)S and functional R group. If polymerization mechanism is proceeded effectively, the rate of the rate of addition/fragmentation equilibrium will be higher than that of the propagation. In other words, less than one monomer unit should be added per activation cycle; resulting in very close degree of polymerization (DP) hence low polydispersity index [50, 51].

RAFT polymerization is a popular technique for the synthesis and design of polymer drug conjugates for variable reasons. It enables to synthesize functional copolymers as well as block polymers. Polymers can be post modified through their chain ends and many organic chemistry strategies are available for this functionalization. As an alternative, the RAFT agents can be modified before polymerization and obtain polymers with functional end groups. These end group functionalized polymers can serve many purposes such as conjugation to bioactive molecules or species [52].

2. AIM OF THE STUDY

This thesis focuses on the design and synthesis of antibody polymer conjugates in order to increase the warhead carried by the antibody, without increasing the number of conjugated sites (Figure 2.1). Moreover, this study aims to establish an analytical method to survey possible conjugation reactions and optimize a reproducible condition. This analytical method(s) should enable calculation of polymer to antibody ratio (PAR). In order to track the polymer attached, copolymers bearing dye molecules are synthesized and later, these copolymers are conjugated to the antibody.

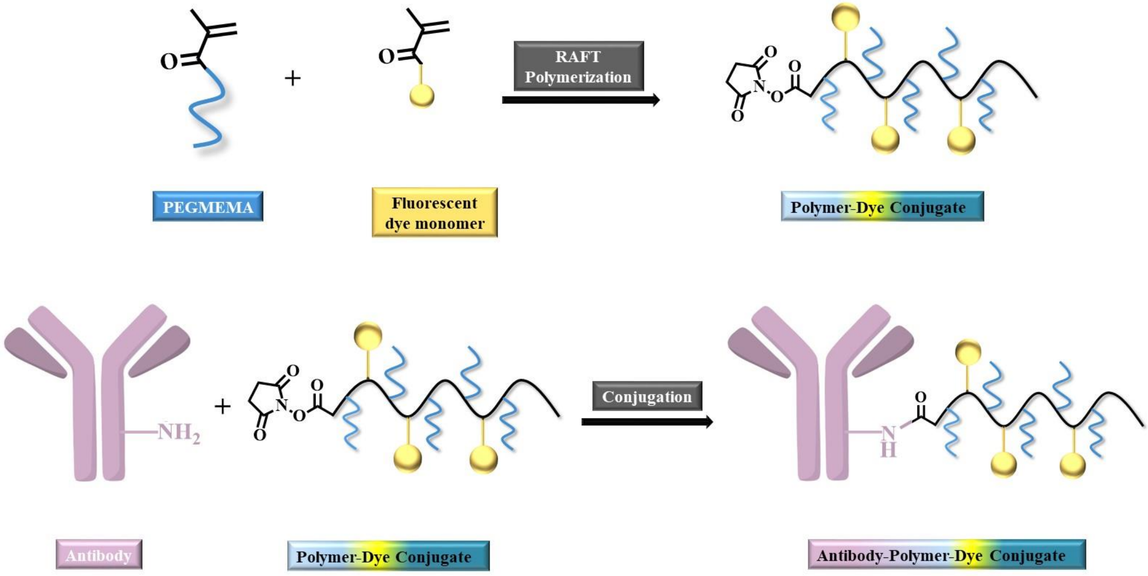


Figure 2.1. General aim of the study

Based on the scope of this project, fluorescein bearing water soluble polymer is synthesized via RAFT polymerization with a modified chain transfer agent and further conjugated to the antibody through the lysine residues on the mAb. As a monoclonal antibody, HER2 targeting trastuzumab is chosen. Activated ester-amine coupling reaction is selected for the conjugation so that no modifications are required on the mAb.

3. EXPERIMENTAL

3.1. Materials and Instrumentation

3.1.1. Chemicals

4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) 2,2'-azobisisobutyronitrile (AIBN), *N,N'*-Dicyclohexylcarbodiimide (DCC) and Fluorescein *O*-methacrylate (FMA) were purchased from Sigma Aldrich. Poly(ethylene glycol) methylether methacrylate ($M_n = 300$) (PEGMEMA, 99%) was also purchased from Sigma Aldrich and it was passed through basic alumina column to remove inhibitor prior to use. N-Hydroxy Succinimide (NHS) was obtained from Alfa Aesar. Herceptin was purchased from Roche. Dialysis membrane MWCO 3.5 kDa was obtained from Spectrum Labs. Millipore ultracentrifge dialysis cassette MWCO 30 kDa was purchased from Merck. All following anhydrous solvents *N,N*-Dimethylformamide (DMF), Acetonitrile (ACN), *N,N*-Dimethylacetamide (DMAC), dichloromethane (DCM) diethyl ether and deuterated chloroform ($CDCl_3$), were obtained from Merck. NaOH and $NaHCO_3$ were purchased from Merck. NaH_2PO_4 was obtained from ABCR. Column chromatography was performed using silicagel–60 (43–60 nm). Thin layer chromatography was performed using silica gel plates (Kiesel gel 60 F254, 0.2mm, Merck).

3.1.2. Instrumentation

SCPDB and all copolymers were characterized via ^{1}H NMR (Avance III HD, 400 MHz, Bruker) and calibrated with respect to solvent signal. The measurements were done in deuterated chloroform ($CDCl_3$). The molecular weights and polydispersity indexes of synthesized copolymers were determined by gel permeation chromatography (GPC, Shimadzu) using PSS-SDV column (Gramlinear, length/ID 8 × 300 mm, 10 mm particle size) calibrated with poly(methylmethacrylate) (PMMA) standards using a refractive-index detector. Dimethylacetamide (DMAc) was used as eluent at a flow rate of 1 mL/min at 30°C. Quantitative analysis of SCPDB was done by LC-MS (Shimadzu) equipped with a column C18 (length/ID 100 x 3 mm, 2.7 µm). Eluted with ACN and ultra-pure water containing 0.05% (v/v) trifluoroacetic acid (TFA), 50-95 gradient flow. Fluorescence spectra of the copolymers and fluorescent methacrylate were measured on a Varian Cary Eclipse spectrophotometer at room temperature. 1 cm path length rectangular quartz cuvette was used. The emission and excitation slit widths are 5 nm. The fluorescent methacrylate emission spectrum was measured with excitation at 512 nm. Purification of conjugates were done by ÄKTA avant Chromatography system (GE Healthcare). Samples were eluted with PBS and ultra-pure water at 1 mL/min flow rate.

3.2. Methods

3.2.1. Synthesis of 4-Cyano-4-(phenylcarbonothioylthio) Pentanoic Succinimide Ester

4-Cyano-4-(phenylcarbonothioylthio) pentanoic succinimide ester (SCPDB) was synthesized according to literature with a slight difference [53]. 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) (200 mg, 0.72 mmol), N-Hydroxy Succinimide (NHS) (91 mg, 0.79 mmol) was dissolved in CH_2Cl_2 (1.5 mL). *N,N'*-Dicyclohexylcarbodiimide (DCC) (163 mg, 0.79 mmol) was dissolved in CH_2Cl_2 (0.5 mL) and added slowly to the reaction mixture in an ice bath under stirring. Reaction was stirred at room temperature for 3 hours. At the end of the reaction, CH_2Cl_2 was removed in vacuo. Minimum amount of CH_2Cl_2 was added and precipitated on cold EtOAc for overnight. Precipitated N,N'-Dicyclohexylurea (DCU) was removed via filtration. Crude was further purified with silica column chromatography (40% v/v EtOAc/Hexane) giving pink solid (141.2 mg, 52% yield). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.93 (d, J = 7.4 Hz, 2H), 7.58 (t, J = 7.4 Hz, 1H), 7.41 (t, J = 7.8 Hz, 2H), 3.03 – 2.95 (m, 2H), 2.86 (s, 4H), 2.79 – 2.69

(m, 1H), 2.61 – 2.52 (m, 1H), 1.96 (s, 3H). LC-MS$_{theo}$ m/z: 376.45 g/mol, LC-MS$_{exp}$ [M+H] m/z: 377 g/mol.

3.2.2. Synthesis of NHS-POEGMEMA (P1)

NHS-POEGMEMA (P1) is synthesized via RAFT polymerization of PEGMEMA where SCPDB is used as chain transfer agent (CTA). PEGMEMA (100 mg, 0.33 mmol) is dissolved in DMF (0.4 mL). Stock solution of SCPDB is prepared in DMF and 0.2 mL (1.79 mg, 4.76 µmol) of this stock solution is added to reaction mixture. Stock solution of AIBN is prepared in DMF and 0.2 mL (0.16 mg, 0.95 µmol) of this solution is added into reaction mixture. Reaction is stirred for 16 hours at 70 °C. After polymerization, solvent is evaporated under *vacuo*. Crude product is precipitated in cold diethyl ether. Diethyl ether is discarded and polymer is obtained as a pink viscous solid (42 mg). (Conversion: 42 %, $M_{n,theo}$: 12600, $M_{n,GPC}$: 27000, $M_{n,NMR}$: 28955, M_w/M_n: 1.4). For polymer P1: ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 4.08 (s, 790H), 3.38 (s, 1158H), 2.85 (s, 4H).

3.2.3. Synthesis of NHS-POEGMEMA-FMA Copolymer (P2)

NHS-POEGMEMA-FMA copolymer (P2) is synthesized via RAFT co-polymerization of PEGMEMA and Fluorescein O-methacrylate (FMA) where SCPDB is used as CTA. PEGMEMA (100 mg, 0.33 mmol), FMA (8.61 mg, 0.022 mmol) and SCPDB (4.05 mg, 0.011mmol) are dissolved in DMF (0.8 mL). Stock solution of AIBN is prepared in DMF and 0.2 mL (0.35 mg, 0.0022 mmol) of this solution is added into reaction mixture. Reaction is stirred for 16 h at 70 °C. After polymerization, DMF is evaporated under *vacuo*. Crude mixture is precipitated in cold diethyl ether. Diethyl ether is discarded and polymer is obtained as an orange-like viscous solid (32.5 mg). (Conversion: 30%, $M_{n,theo}$: 3030, $M_{n,GPC}$: 13000, $M_{n,NMR}$: 12800, M_w/M_n: 1.3). (Obtained ratio [PEGMEMA]:[FMA]= 40:2, [FMA]/[P2]= 0.84). For polymer P2: ^{1}H NMR (400 MHz, CDCl3, δ, ppm) δ 8.01 (s, 3H), 4.27 – 3.96 (m, 122H), 3.38 (s, 184H), 2.84 (s, 4H).

3.2.4. Conjugation of Trastuzumab with NHS-POEGMEMA-FMA

Conjugation reactions are carried out based on literature with some modifications [54, 55]. In general, Trastuzumab (Tz) and polymer are dissolved in buffer and stirred 25 °C for 18 h. As an example, C1-P2 conjugate reaction is explained in detail; others are set up by following same procedure. Tz (0.5 mg, 0.0035 µmol) and P2 polymer (0.9 mg, 0.069 µmol) are dissolved in 0.1M Bicarbonate buffer (0.9 mL).

When the reaction is over, in order to get rid of excess polymer; dialysis is carried out with Millipore dialysis cassette 30kDa cut-off. Further purification is done via hydrophobic interaction column chromatography (HIC). Polymer/Tz ratios are obtained as following: C4-P2 = 1.69, C6-P2 = 2.92, C8-P2 = 2.56, C9-P2 = 2.36.

3.2.5. Characterization of Trastuzumab-POEGMEMA-FMA

Firstly, FMA standard samples (0.050 ppm, 0.10 ppm, 0.25 ppm and 0.50 ppm concentrations) are prepared in 1.0 N NaOH. Fluorescence intensities of these samples are measured in fluorescence spectroscopy and calibration curve is drawn. In order to calculate polymer to antibody ratio (PAR), fluorescence intensities of polymers and conjugates are measured. By using the calibration curve, FMA concentrations in polymers and conjugates are calculated. Lastly, these values are placed in following equation and PAR values are calculated.

$$PAR = \frac{FMA/Tz}{FMA/Polymer} \tag{2.1}$$

4. RESULTS AND DISCUSSION

In order to design and optimize the antibody-polymer–dye conjugate system, polymers containing fluorescent dye (FMA) were synthesized and conjugated to antibody. Moreover, these polymers synthesized to have NHS-ester functional group at the end of polymer chains in order to conjugate them to trastuzumab through lysine amino acid via stable amide bond formation. RAFT is used as a polymerization technique which requires use of chain transfer agent (CTA). Polymers were grown between the parts of CTA molecule due to the mechanism of RAFT polymerization. This means that end group of the polymers are defined by the chosen CTA molecule. Based on these information, CPADB molecule, which serves as a CTA, was activated via esterification reaction with NHS to obtain NHS-ester functional group at the end of the molecule hence at the end of the polymer chains which were synthesized by using this molecule.

Dye/polymer ratios were calculated both in terms of concentration as well as in terms of number of molecule per polymer chain. These values were used to calculate polymer to antibody ratio (PAR). Results indicate that conjugating polymers to antibody, increases the number of dye molecule carried by one antibody. This is a promising result because it indicates that number of payload can be increased without increasing the number of conjugated sides.

4.1. Characterization of SCPDB

SCPDB molecule was synthesized via esterification reaction by the help of commonly used coupling reagent DCC and obtained with a 52% yield (Figure 4.1).

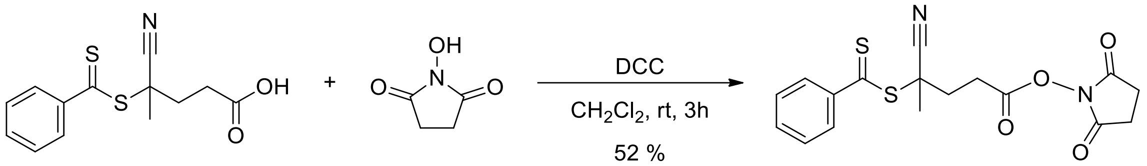


Figure 4.1. Synthesis of SCPDB molecule

^{1}H NMR spectra of SCPDB clearly shows the existence of NHS ester functional group via characteristic peak of NHS protons namely g at 2.86 ppm. Protons a, b and c belongs to the benzene ring on the molecule which are located at 7.93, 7.58 and 7.41 ppm respectively. Detailed characterization is shown in Figure (4.2).

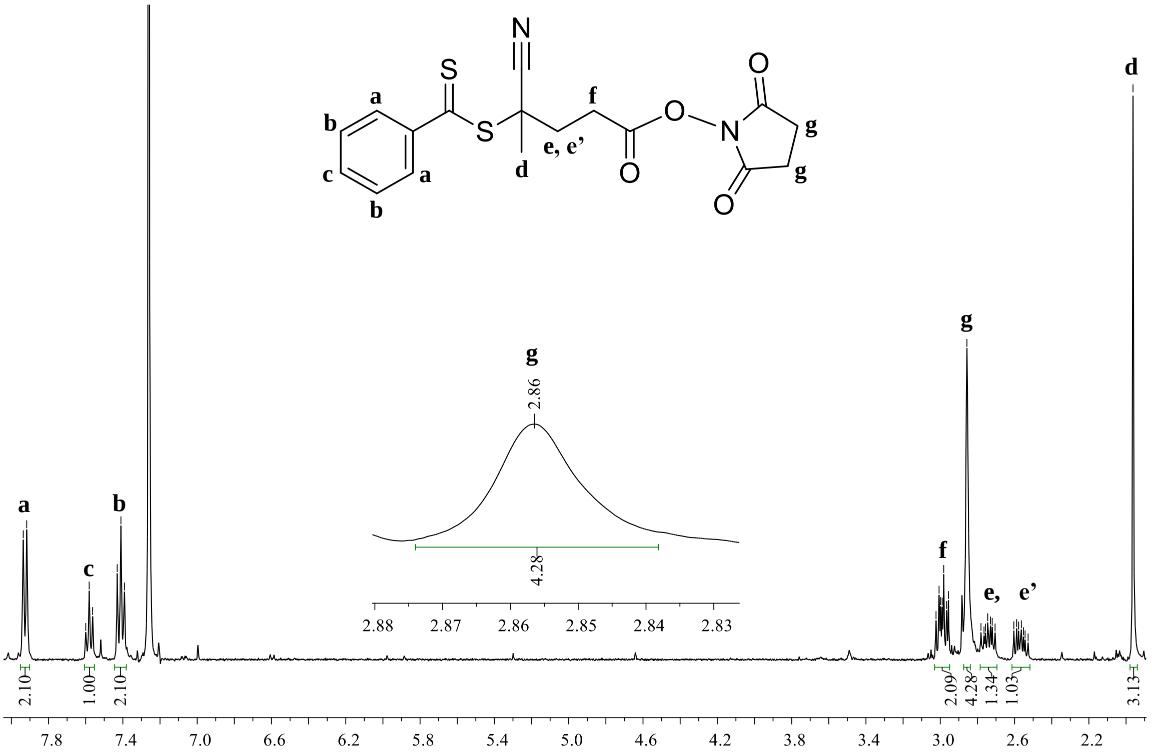


Figure 4.2. ^{1}H NMR spectra of SCPDB molecule in $CDCl_3$

4.2. Synthesis and Characterization of NHS-POEGMEMA Polymer

NHS esters are also known as activated esters meaning they are ready to react with any nucleophile in the medium. Considering this information along with the aim of testing the efficiency of SCPDB in polymerization reaction, NHS-POEGMEMA polymer (P1) is synthesized (Figure 4.3).

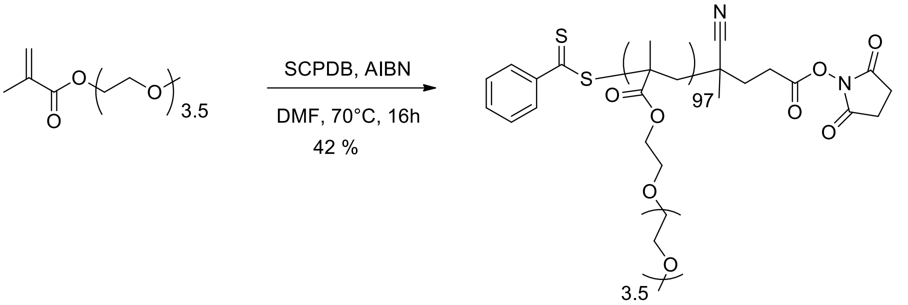


Figure 4.3. Synthesis of NHS-POEGMEMA polymer (P1)

Existence of NHS-ester group at the end of the polymer chain was confirmed via ^{1}H NMR spectrum (Figure 4.4).

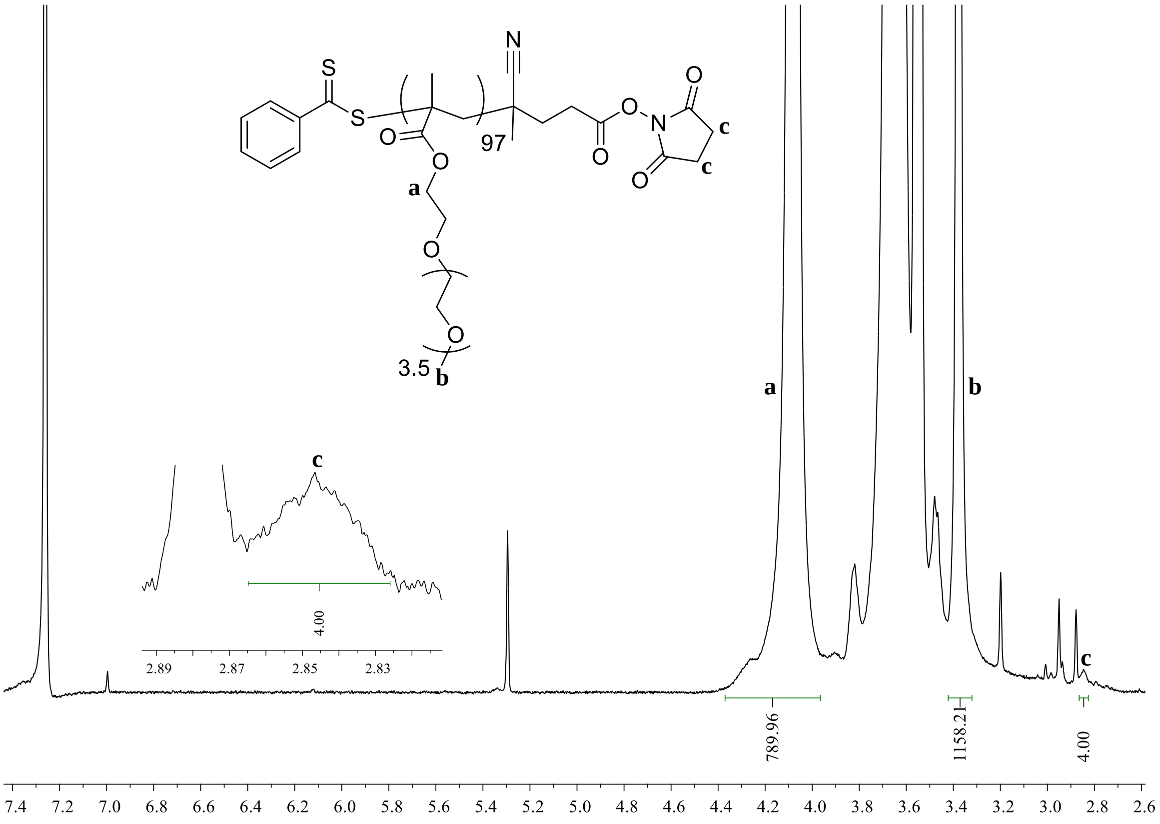


Figure 4.4. ^{1}H NMR spectra of P1 in $CDCl_3$

Proton named a gives signal at 4.08 ppm which belongs to O-CH_2 ester protons of PEGMEMA and proton b at 3.38 ppm belongs to O-CH_3 eter protons of PEGMEMA. Repeated unit was calculated by the ratio of molecular weight of the P1 to the integral values of these signals which resulted in average 97 PEG chains per polymer chain. NHS-ester protons named c give signal at 2.85 ppm shows that polymer chains bears the functional group as it was aimed.

4.3. Synthesis and Characterization of NHS-POEGMEMA-FMA Copolymer

In order to calculate PAR after conjugation with Tz, quantitative analysis method was required. For this reason, dye monomer FMA was introduced to polymer reaction. Polymerization reactions were carried via RAFT polymerization technique where SCPDB was used as CTA and AIBN was used as radical initiator (Figure 4.5).

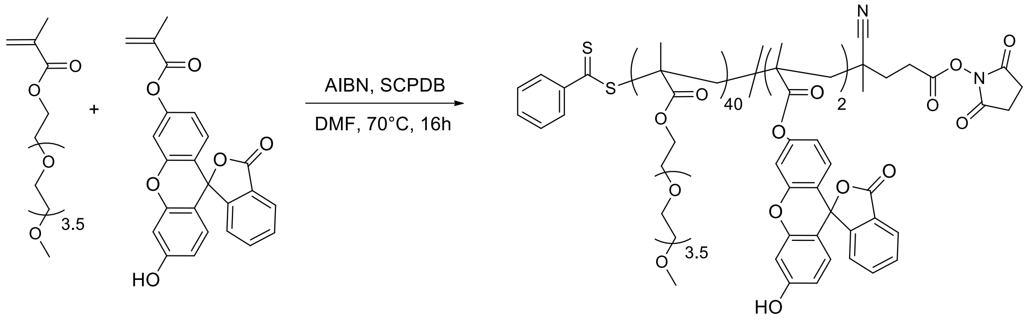


Figure 4.5. Synthesis of NHS-POEGMEMA-FMA copolymer P2

Detailed reaction conditions and results are given Table 4.1.

Table 4.1. Polymerization conditions and characterizations of copolymer P2

No	Polymer	Feed Ratio $[M1]:[M2]^a$	Obtained Ratio $[M1]:[M2]^{a,b}$	$M_{n,\ theoretical}$ (Da)	GPC^c Results		$M_{n,\ NMR}{}^b$ (Da)	$[FMA]/[Polymer]^d$	Conversion (%)
					M_n (Da)	M_w/M_n			
1	P2	[31]:[2]	[40]:[2]	10100	13000	1.3	12800	0.84	30%

^{a}M1:PEGMEMA, M2:FMA, [SCPDB]:[AIBN] = 5:1, ^{b}calculated via integration values in ^{1}H NMR spectra, ^{c}obtained by GPC eluted with DMAC, relative to linear PMMA standards, ^{d}calculated via intensity values obtained from fluorescence spectroscopy based on FMA calibration curve.

Synthesized copolymer (P2) were characterized via ^{1}H NMR spectra (Figure 4.6). For copolymer P2**,** proton named c, belongs to the dye molecule, particularly the benzene ring which is fused to γ-lactone ring. This causes downfield shift of the benzene proton peaks to 8.01 ppm. Existence of this peak proves that copolymerization with FMA was successfully done. Moreover, signal at 2.84 ppm proves that copolymer P2 has its end group as NHS-ester functional group. Repeating units were calculated from the integration values of the ^{1}H NMR peaks. From this calculation, number of monomers (PEGMEMA:FMA) in one polymer chain were obtained as 40:2 for copolymer P2.

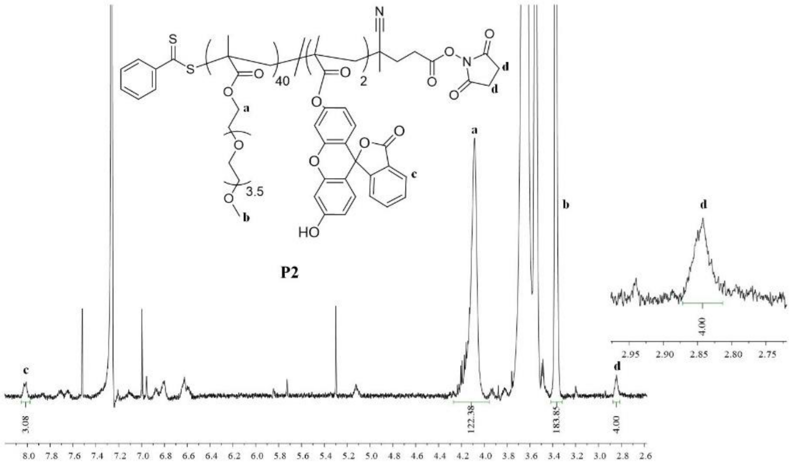


Figure 4.6. ^{1}H NMR spectra of NHS-POEGMEMA-FMA copolymer (P2) in $CDCl_3$

It was aimed to find [FMA]/[Polymer]. For this reason, FMA calibration curve was drawn by using fluorescence spectroscopy (Figure 4.7).

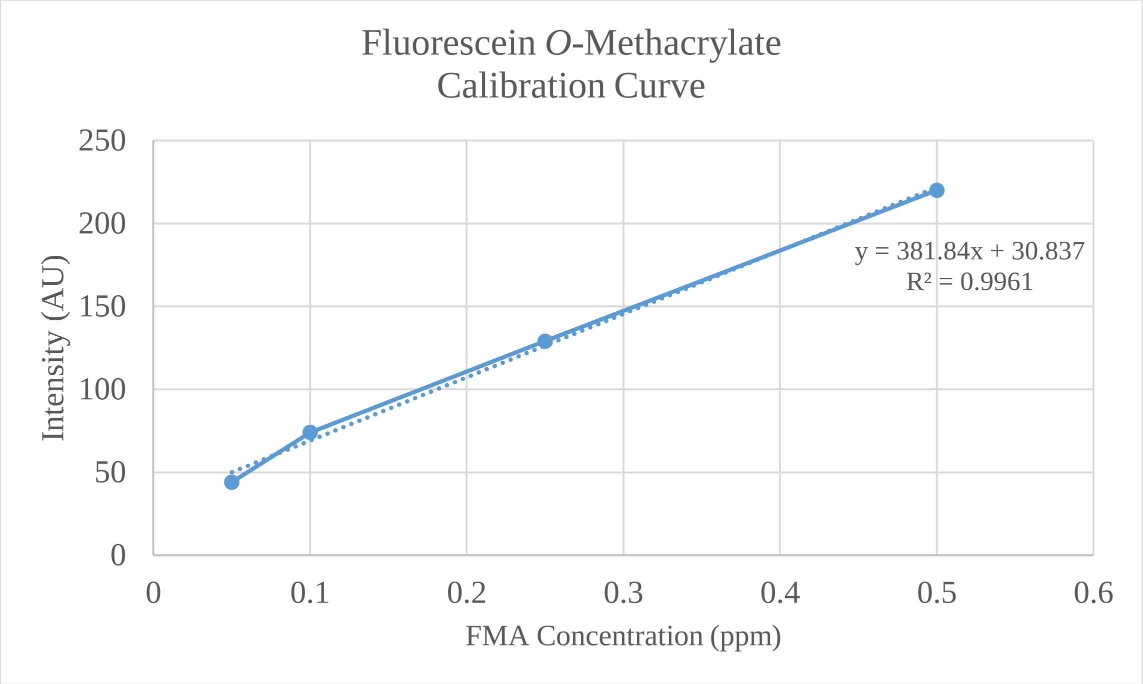


Figure 4.7. FMA Calibration Curve

For each polymer, dye concentrations were calculated from this calibration curve and converted to molarities. Moreover, polymer concentrations were calculated in molarities and finally these values are divided to find dye per polymer (Equation 2.2).

$$Dye\ per\ Polymer = \frac{[FMA]}{[Polymer]} \qquad (2.2)$$

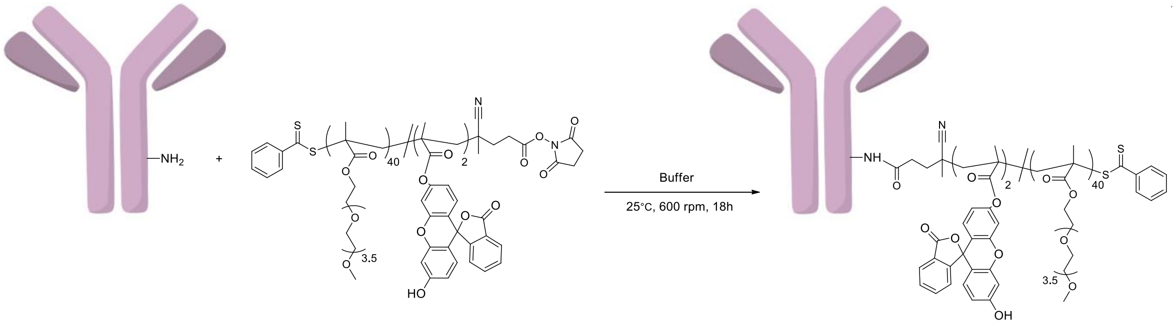
From this equation dye per polymer values were calculated as 0.84 for copolymer P2.

4.4. Conjugation of Trastuzumab with NHS-POEGMEMA-FMA and Purification of Conjugates

Antibody-polymer-dye conjugation was based on amine-ester reaction yielding an amide bond between the antibody and polymer. To obtain this architecture, polymers with

NHS-ester end groups were conjugated to commercially available trastuzumab (Tz) with slight modifications on literature examples by trial and error [54, 55].

In this section, 9 conjugations were performed and general synthesis was shown in Figure 4.8. Conjugates were numbered as CX-P2 where CX is the number of conjugate and P2 is the polymer which was used in that conjugation.



CX-P2 where X: 1 to 9

Figure 4.8. General synthesis of Trastuzumab-POEGMEMA-FMA conjugates

Details of reaction conditions were shown in Table 4.2.

Table 4.2. Trastuzumab-Polymer conjugation reaction conditions

No	Conjugate	Feed Ratios (Tz:Polymer)	Buffer	pH	Final Polymer Concentration [M]
1	C1-P2	1:20	0.1 M Bicarbonate	8.4	7.7×10^{-5}
2	C2-P2	1:20	0.1 M Bicarbonate	8.4	7.7×10^{-5}
3	C3-P2	1:20	0.1 M Bicarbonate	8.4	7.7×10^{-5}
4	C4-P2	1:10	0.1 M Bicarbonate	8.4	7.7×10^{-5}
5	C5-P2	1:10	0.1 M Bicarbonate	8.4	7.7×10^{-5}
6	C6-P2	1:20	PBS	7.4	7.7×10^{-5}
7	C7-P2	1:20	PBS	7.4	7.7×10^{-5}
8	C8-P2	1:20	PBS	7.4	7.7×10^{-5}
9	C9-P2	1:20	PBS	7.4	7.7×10^{-5}

Synthesis of conjugates CX-P2 where X is 1 to 5 were shown in Figure 4.9. For these conjugates 1 to 5, buffer was 0.1 M Bicarbonate.

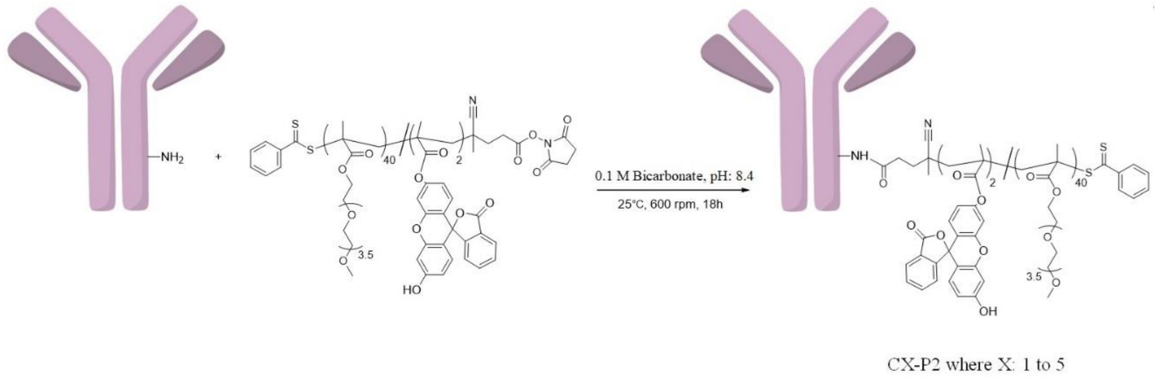


Figure 4.9. Synthesis of conjugates CX-P2 where X: 1 to 5

In order to see the effect of buffer on conjugation efficiency, conjugates CX-P2 (X: 6 to 9) were synthesized (Figure 4.10).

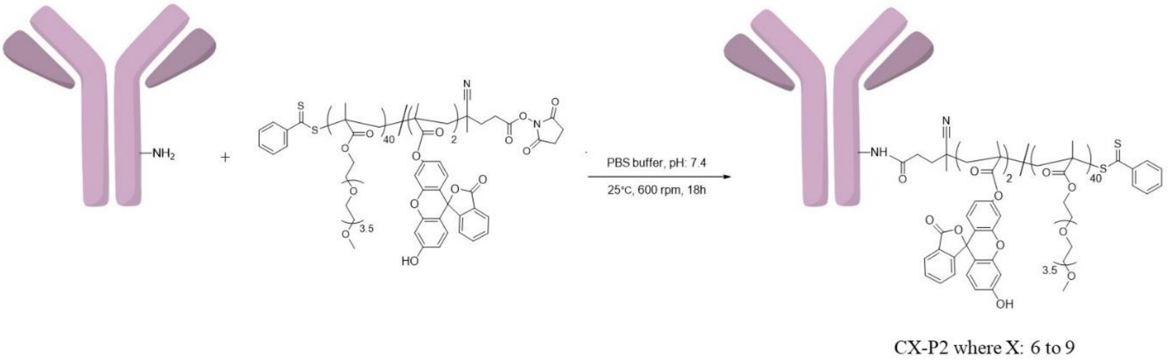


Figure 4.10. Synthesis of conjugates CX-P2 where X: 6 to 9

For all conjugates, same purification method was applied. When reaction is over, excess polymer was removed from the media via centrifuge and for this process 30 kDa cut-off Millipore dialysis cassettes were used. Centrifuge was continued until filtrate was free of polymer which was controlled via UV absorbance at FMA specific wavelength (at 496 nm). In order to get rid of unreacted Tz, further purification was done via hydrophobic interaction column (HIC) connected to Äkta Avant chromatography system. Elution was based on salting out principle. Example chromatogram result is shown in Figure 4.11.

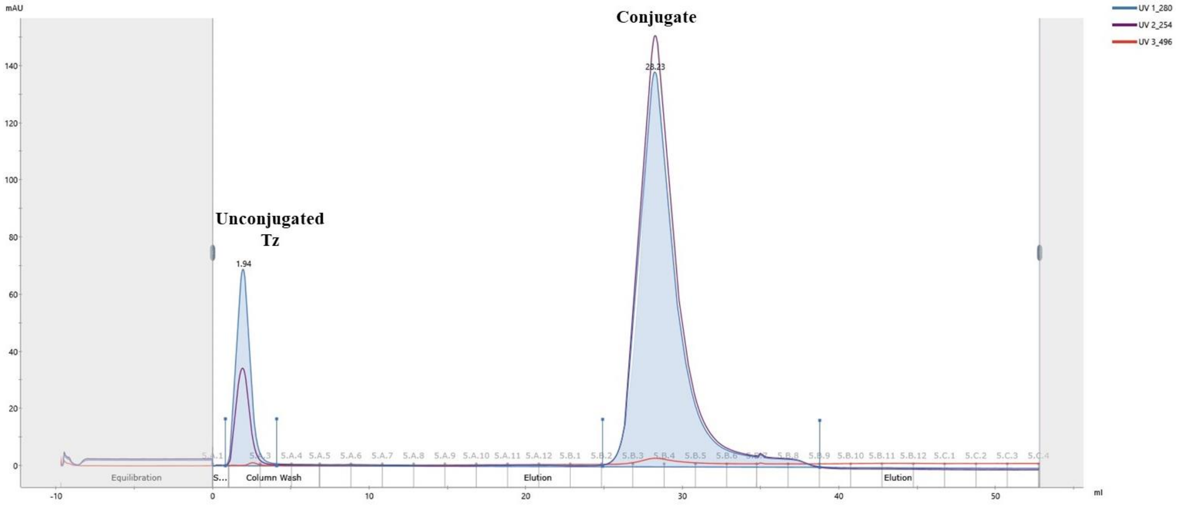


Figure 4.11. Example HIC Chromatogram of conjugates

4.5. Characterization of Trastuzumab-POEGMEMA-FMA Conjugates

After purification, characterization of conjugates was done by following several measurements on fluorescence spectroscopy and UV spectroscopy followed by calculations using equation 2.1 and 2.2.

In order to see the effect of feed ratio as well as to see repeatable results, conjugates C1-P2 to C5-P2 were synthesized. To have an insight idea about PAR and also to prove the increase on PAR before and after HIC purification where unreacted Tz was removed from conjugates, fluorescence spectroscopy measurements were performed after dialysis. Results were shown in Table 4.3. Considering C1-P2 to C3-P2, they have same feed ratio and similar preliminary PAR values on average 0.98. This shows that system gives repeatable results. Considering conjugates C4-P2 and C5-P2, they have also same feed ratio and similar PAR values. However, it was expected to see difference in preliminary PAR values in relation with feed ratio when compare the conjugates 1-3 to 4-5. Having similar preliminary PAR values may be resulted from calculating these results before going further purification meaning samples were containing unreacted Tz which affects the PAR ratio.

Table 4.3. Preliminary results of conjugates C1-P2 to C5-P2

No	**Conjugate**	**Feed Ratio (Tz:Polymer)**	**Buffer**	**Preliminary PAR Values (After Dialysis)**
1	C1-P2	1:20	0.1 M Bicarbonate	0.97
2	C2-P2	1:20	0.1 M Bicarbonate	1.00
3	C3-P2	1:20	0.1 M Bicarbonate	0.97
4	C4-P2	1:10	0.1 M Bicarbonate	0.86
5	C5-P2	1:10	0.1 M Bicarbonate	0.75

It was aimed to see effect of buffer on conjugation efficiency so conjugates C6-P2 and C7-P2 were synthesized (Figure 4.10).

For the same reason explained above, their PAR calculation done after dialysis without continuing further purification. Results were shown in Table 4.4. Conjugates have same feed ratio with the conjugates no 1 to 3 shown in Table 4.3. Preliminary PAR results were obtained as 1.92 and 1.26 for C6-P2 and C7-P2 respectively. These values also higher than conjugates C1-P2 to C3-P2 and prove the effect of buffer on conjugation efficiency.

Table 4.4. Preliminary results of conjugates C6-P2 and C7-P2

No	**Conjugate**	**Feed Ratio (Tz:Polymer)**	**Buffer**	**Preliminary PAR Values (After Dialysis)**
1	C6-P2	1:20	PBS	1.92
2	C7-P2	1:20	PBS	1.26

Based on these results, it was decided to carry on further purification of conjugates C4-P2 and C6-P2 with HIC chromatography. Results were shown in Table 4.5. For both conjugates, effect of further purification on PAR was observed clearly. Their PAR were increased to 1.69 and 2.92 from 0.86 and 1.92 respectively. Moreover, effect of buffer on conjugation efficiency was proved certainly. Based on these results, further conjugations are performed in PBS buffer.

Table 4.5. Preliminary and Final Results of Conjugates C4-P2 and C6-P2

No	**Conjugates**	**Buffer**	**Preliminary PAR Values (After Dialysis)**	**Final PAR Values (After HIC Chromatography Purification)**
1	C4-P2	Bicarbonate	0.86	1.69
2	C6-P2	PBS	1.92	2.92

In order to see repeatability of this system C8-P2 and C9-P2 conjugates were synthesized with the same conditions of C6-P2. After all purification steps were completed, their PAR values were calculated. Results were shown in Table 4.6. Upon repeated synthesis, PAR results were close to each other and on average it is 2.61. Polymer P2 that was used for this conjugate, carries 2 dye molecule per chain. From this information, average 5.2 dye molecule were carried by these conjugates. It shows that, even if these mild conditions, these systems have capacity to carry more agents than on the market.

Table 4.6. Results of conjugates C6-P2, C8-P2 and C9-P2

No	**Conjugates**	**Final PAR Values (After HIC Chromatography Purification)**	**Number of Dye Molecule carried by one Tz**
1	C6-P2	2.92	5.84
2	C8-P2	2.56	5.12
3	C9-P2	2.36	4.72

5. CONCLUSIONS

This thesis focuses on the design of antibody polymer dye conjugates. In summary, the aim was finding an analytical method which does not effected by the polydispersity of the analyzed material. Based on this approach, polymers with modified end group have been synthesized. In order to find how to calculate polymer to antibody ratio (PAR) as well as to optimize the conjugation conditions, trastuzumab was conjugated to fluorescent dye containing copolymer. Dye concentrations in polymer as well as conjugates were determined via putting the fluorescence intensity values into FMA calibration curve.

Under the light of obtained results the following conclusions can be drawn:

- SCPDB, which is a CTA molecule with NHS-ester end group, was synthesized successfully.
- Using SCPDB, copolymer with PEGMEMA and FMA was synthesized. End group of the polymer chains was kept as NHS-ester which was ensured by the mechanism of RAFT polymerization.
- Conjugation with trastuzumab was done via stable amide bond formation between the NHS-ester end group of polymers and NH_2 group on lysine side chain. By this chemistry, integrity of trastuzumab was not disturbed since it does not include breaking the antibody to its part by reducing the S-S bridges like in literature examples [37, 45, 49].
- Moreover, amide bonds are stable in physiological media, they require specials enzymes to break down. For this reason, this chemistry prevents releasing polymer from antibody.
- Efficiency of conjugation severely depends on choice of buffer. By comparing Table 4.4 and Table 4.5 it has concluded that PBS is a better buffer for these reactions.
- Conjugates performed with NHS-POEGMEMA-FMA copolymer, indicates that higher DAR values can be obtained as aimed.

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APPENDIX A: ^{1}H NMR SPECTROSCOY DATA

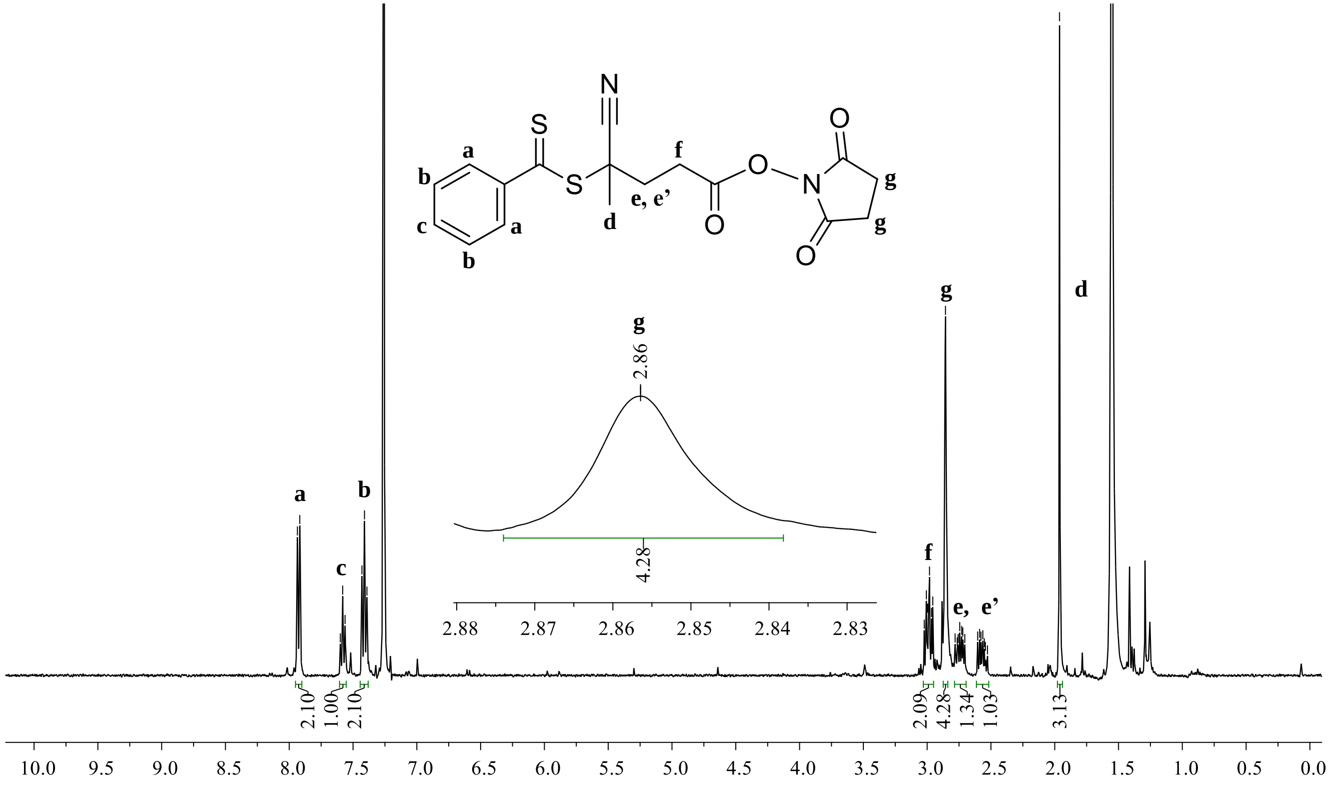


Figure A.1. ^{1}H NMR spectrum of SCPDB molecule ($CDCl_3$)

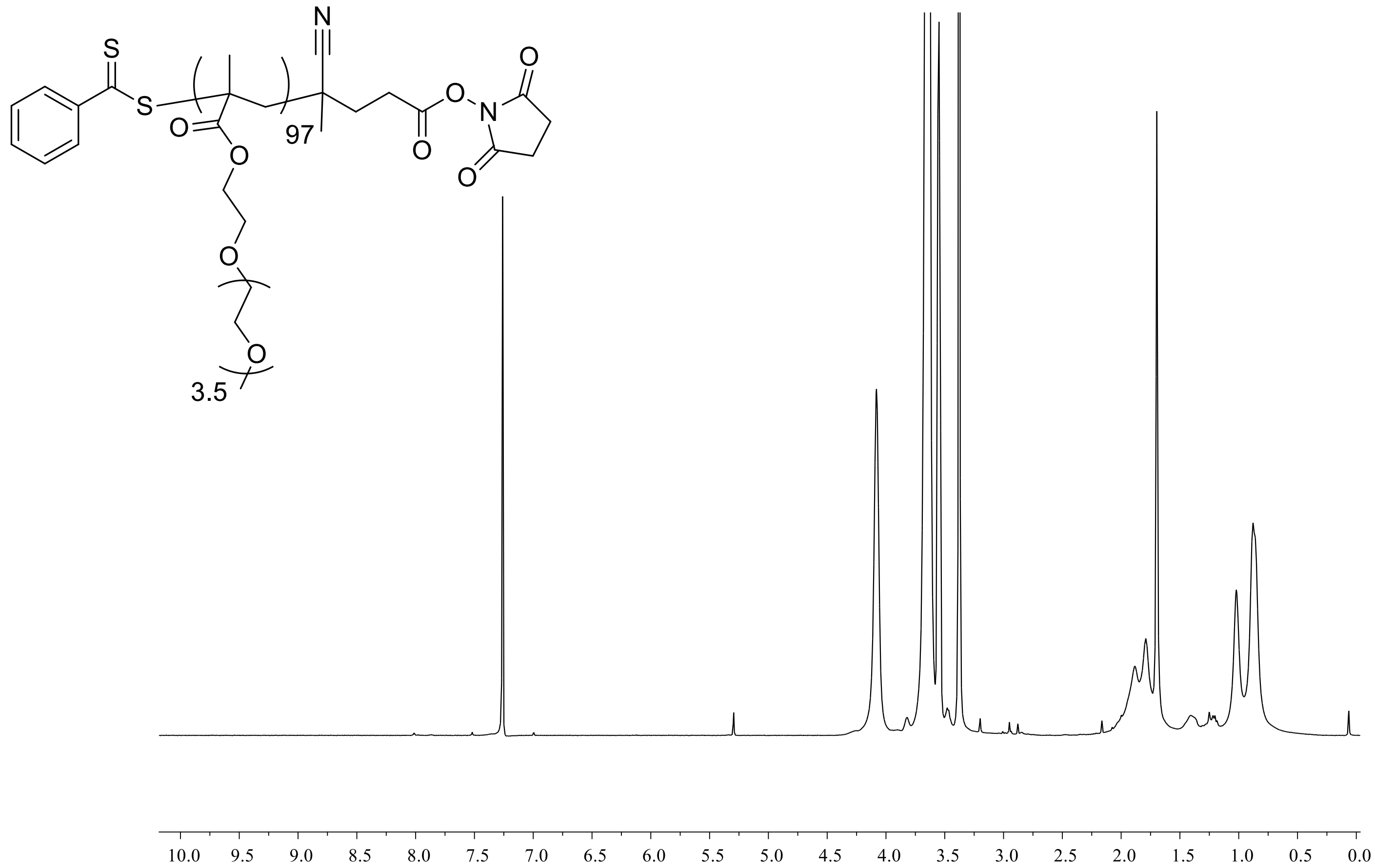


Figure A.2. ^{1}H NMR spectrum of NHS-POEGMEMA polymer (P1) (CDCl$_3$)

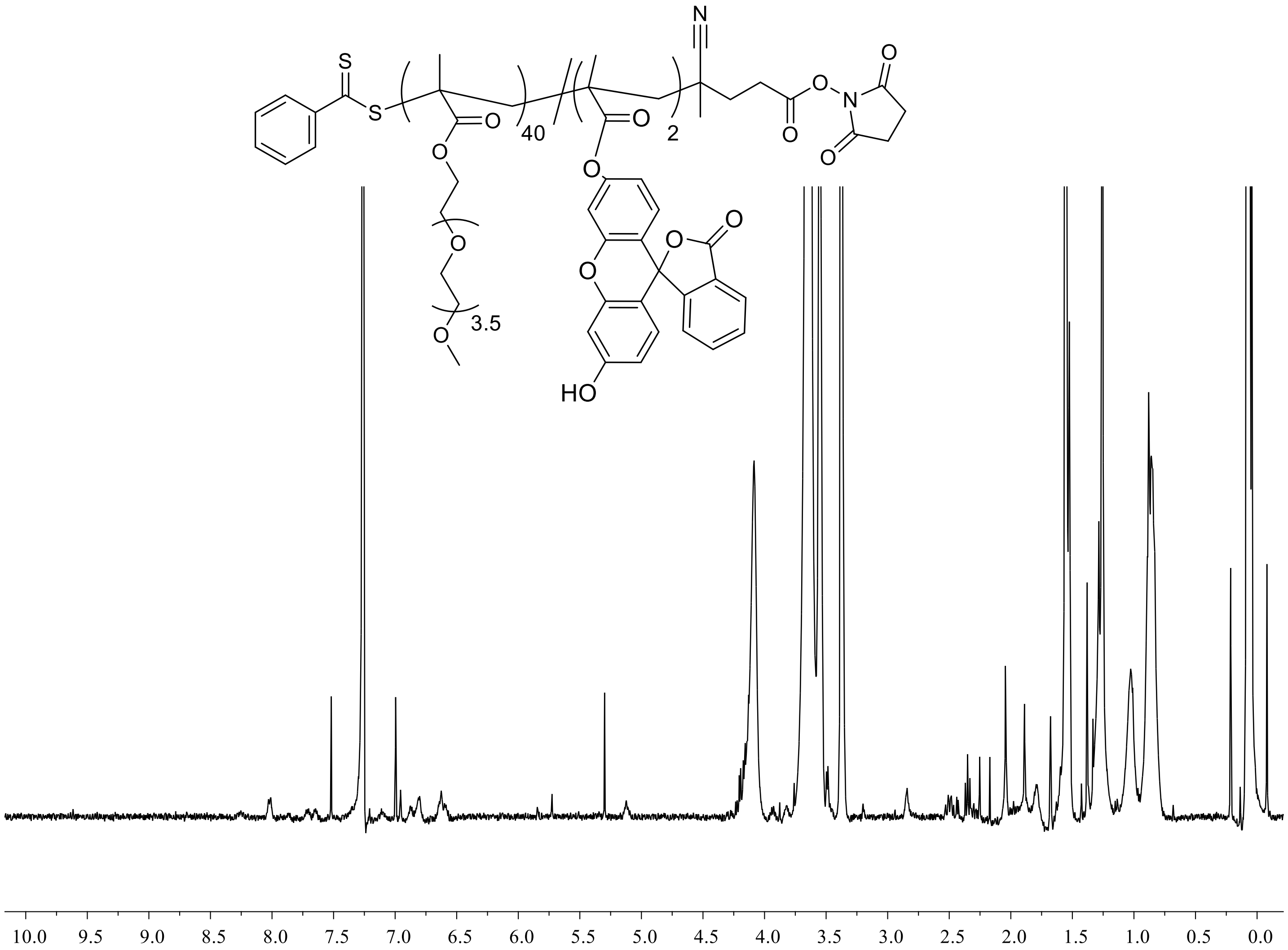
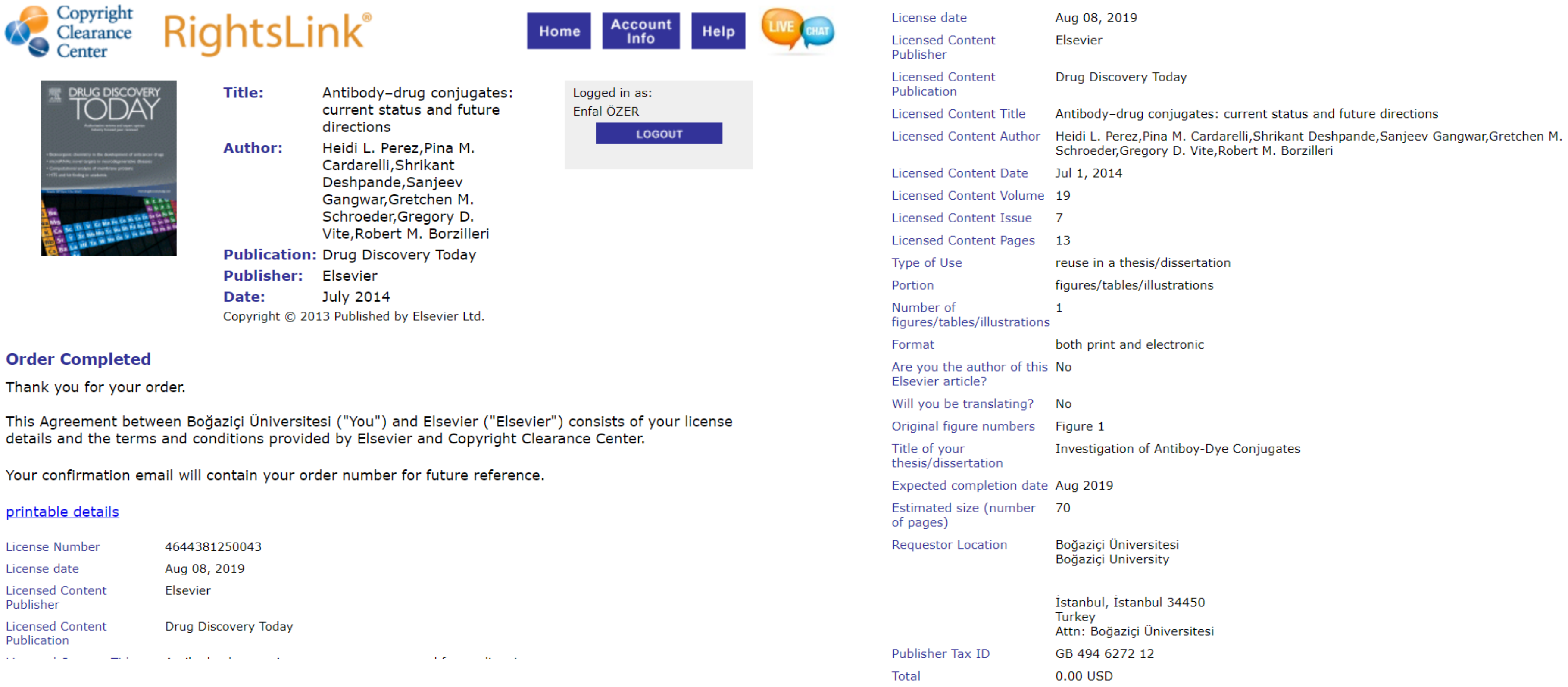


Figure A.3. ^{1}H NMR spectra of NHS-POEGMEMA-FMA copolymer (P2) in $CDCl_3$

APPENDIX B: COPYRIGHT NOTICES



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