ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

PREPARATION OF FUNCTIONAL SURFACES AND PROTEIN POLYMER CONJUGATES VIA LIGHT INDUCED TETRAZINE CLICK REACTION

M.Sc. THESIS

Nagihan ÖZBEK

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

IŞIKLA TETİKLENEBİLİR TETRAZİNE KLİK REAKSİYONU İLE FONKSİYONEL YÜZEYLERİN VE PROTEİN POLİMER KONJUGATLARININ HAZIRLANMASI

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Nagihan ÖZBEK, a M.Sc. student of ITU Graduate School of Science Engineering and Technology student ID 509171234, successfully defended the thesis/dissertation entitled "PREPARATION OF FUNCTIONAL SURFACES AND PROTEIN POLYMER CONJUGATES VIA LIGHT-INDUCED TETRAZINE CLICK REACTION ", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family and friends,

FOREWORD

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June 2020

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TABLE OF CONTENTS

Page

ABBREVIATIONS

Tz : Tetrazine
dHTz : Dihydrotetrazine
Nb : Norbornene
DA : Diels-Alder reaction
DCC : N,N'-Dicyclohexylcarbodiimide
TCO : 5-hydroxy-1-cyclooctene (transcyclooctenol)
AIBN : 2,2′-Azobis(2-methylpropionitrile)
DMF : Dimethylformamide
DMSO : Dimethylsulfoxide
NIPAAm : N-isopropylacrylamide
CTA : Chain Transfer Agent
RAFT : Reversible Addition-Fragmentation Chain-Transfer
ATRP : Atom Transfer Radical Polymerization
ROP : Ring-Opening Polymerization
PNIPAAm : Polynipam/ Poly(N-isopropylacrylamide)
IEDDA : Inverse Electron Demand Diels-Alder Reaction
BSA : Bovine Serum Albumine
LCST : Lower Critical Solution Temperature
Con A : Concovaline A (jackbean) protein
PPC : Protein-polymer conjugates
^{1}H-NMR : Nuclear Magnetic Resonance Spectroscopy
WCA : Water Contact Angle
FT-IR : Fourier Transform Infrared
GPC : Gel Permeation Chromatography
SEM : Scanning Electron Microscope
MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization Spectrometry
PDI : Polydispersity Index
LED : Light-emitting diode

SYMBOLS

M	**:** molar
µM	**:** mikromolar
mmol	**:** milimol
˚C	**:** Celsius
eq.	**:** equivalent
Mn	**:** The number average molecular weight
Mw	**:** The weight average molecular weight
Mw/Mn	**:** Polydispersity Index

LIST OF TABLES

Page

LIST OF FIGURES

Page

PREPARATION OF FUNCTIONAL SURFACES VIA LIGHT INDUCED TETRAZINE CLICK REACTION

SUMMARY

Click reactions have been essential tool for fabrication and modification of functional materials from the beginning of the twenty-first century. After this milestone, several click approach including cycloaddition reactions (Huisgen 1,3-dipolar cycloaddition, also the Diels-Alder (DA) reactions), nucleophilic ring-opening reactions, reactions of some carbonyl compounds, thiol-ene chemistry and Michael additions have been developed. The valuable features including quantitative yields under mild conditions, not producing offensive byproducts and tolerant to many chemical functionalities make this chemistry elegant toolbox in material science and macromolecular engineering.

Additionally, some of these reactions are employed in chemical biology and considered as bioorthogonal reactions, since they can occur inside of living systems without interfering with native biochemical processes. Amongst these reactions, Inverse Electron Demand Diels-Alder (IEDDA) Reaction has come into prominence recently, due to its remarkable kinetics and biorthogonality.

Polymer chemists endeavor the synthesis of macromolecules, which have advanced architectures (e.g. homopolymers and block copolymers). Combinations of DA "click" reactions with live/controlled polymerization methods are effective solutions to create various architectures, which qualify reversible added fragmentation chain transfer polymerization (RAFT), nitroxide-mediated radical polymerization, atom transfer radical polymerization (ATRP), ring-opening polymerization (ROP).

With its high selectivity and super-fast kinetics, the tetrazine-mediated IEDDA Click Reaction is an essential modification technique, which is used in this thesis study. The addition of tetrazine portions to the polymer chains requires a post-polymerization step leading to multi-stage synthetic routes and functional polymeric materials. Such approaches result in the decomposition of the polymer and the molecule, which are specified for modification.

In this study, the modification of glass surfaces was made by using the light-induced version of the DA reaction. The molecule required in this reaction was obtained by photooxidation of the tetrazine molecule. Therefore, two distinct strategies were employed; ***(1)*** grafting dHTz functional substrate (i.e. polymer) onto *trans*-5-hydroxycyclooctene (TCO)-modified surfaces and ***(2)*** grafting TCO-functional substrate (i.e. protein) onto dHTz-modified surfaces. In the first strategy, glass surfaces were decorated with TCO molecules by a typical silanization with 3-aminopropyltriethoxysilane (APTES) followed by straightforward transformations, while PNIPAAm synthesized by RAFT polymerization, was functionalized with a dHTz derivative (PPA-dHTz) by amidation at carboylic acid terminal of the polymer. Then, dHTz functional PNIPAAm was grafted to TCO functionalized glass surfaces by *in situ* photochemical transformation of the dihydrotetrazine to tetrazine and consequent IEDDA Click Reaction.

In the second strategy, glass surfaces were functionalized with dHTz, to which proteins containing TCO moiety was grafted via photo-IEDDA. In both cases, glass surfaces were characterized by water contact angle measurements, SEM and FT-IR

spectroscopy analyses**.** The key point here is to ensure that the oxidation step during the conversion of dHTz to tetrazine occurs by photochemically by click / conjugation reaction.

With this study, it is propounded to develop a method that will enable the modification of the material surface in advanced technological applications, and that glass surfaces will be modified for the first time with a fast, effective, light-induced tetrazine click reaction. Also, we applied this method on the preparation of protein-polymer conjugates (PPC). These conjugates are prepared by performing a light-induced IEDDA reaction with the help of the tetrazine molecule formed under the light with *trans*-cyclooctene (TCO), which is the partner molecule of the reaction, and polymer bonding. Similarly, dHTz functional PNIPAAm was prepared.

On the other hand, proteins (Concanavalin A and bovine serum albumin, BSA) were functionalized with TCO molecule in the PBS medium. Finally, dHTz functional PNIPAAm and TCO functional proteins were conjugated via light-induced IEDDA. The same strategy of preparation of functionalized glass surface have been used for preparation of PPC, in which the oxidation step during the conversion of dHTz to tetrazine takes place by a photochemical reaction. Finally, the PPCs were analyzed with mass spectroscopy (MALDI-TOF) and this ongoing method could be improved in future studies.

IŞIKLA TETİKLENEBİLİR TETRAZİNE KLİK REAKSİYONU İLE FONKSİYONEL YÜZEYLERİN HAZIRLANMASI

ÖZET

"Click" (Klik) reaksiyonları, yirmi birinci yüzyılın başlarından itibaren fonksiyonel malzemelerin üretimi ve modifikasyonu için çok önemli bir reaksiyon haline gelmiştir. Bu tarihi denemeden sonra, siklokatılma reaksiyonları (Huisgen 1,3 - dipolar siklokatılma ve ayrıca Diels-Alder(DA) reaksiyonları), 2-4 nükleofilik halka açılma reaksiyonları, bazı karbonil bileşiklerinin reaksiyonu, tiol-ene kimyası ve Michael katılmaları geliştirilmiştir. Ilımlı koşullar altında kantitatif verimleri içeren, herhangi bir rahatsız edici yan ürün üretmeyen ve birçok kimyasal işlevselliğe toleranslı olan değerli özellikler, bu kimyayı malzeme bilimi ve makromoleküler mühendislikte mükemmel bir araç haline getirir.

Ayrıca, bu reaksiyonların bazıları kimyasal biyolojide kullanılır ve doğal biyokimyasal süreçlere müdahale etmeden canlı sistemlerin içinde meydana gelebildikleri için biyo-ortogonal reaksiyonlar olarak kabul edilir. Ters elektron gereksinimli Diels-Alder (IEDDA) reaksiyonu, kayda değer kinetiği ve biyoortogonal özelliği nedeniyle son zamanlarda öne çıkmıştır. IEDDA [4 + 2] siklokatılma reaksiyonu, elektronca zengin bir dienofil (çoğunlukla gergin halkalı olefin) ve elektron bakımından fakir bir dien (çoğunlukla 1,2,4,5-tetrazin)olmak üzere iki öncü molekül arasında köprü oluşmasıyla ve azot gazı salımıyla altı üyeli bir halka verecek şekilde gerçekleşir.

Tetrazin her zaman iki bileşenden biri olduğundan, reaksiyona bazen tetrazin ligasyonu denir. İkinci bileşen en reaktif substrat molekülü olan *trans*-siklookten türevi olduğunda, kantitatif verim saniyeler ila dakikalar arasında elde edilir. Polimer kimyacılarının amaçlarından biri, ileri mimarilere sahip (örneğin homopolimerler, telekelik, dendronize ve yıldız polimerler ve blok ve aşı kopolimerleri) olan makromoleküllerin sentezidir.

Yaşayan / kontrollü polimerizasyon yöntemleri (tersinir ilaveli parçalanma zinciri transfer polimerizasyonu (RAFT), nitroksit aracılı radikal polimerizasyon (NMRP), atom transfer radikal polimerizasyonu (ATRP), halka açma polimerizasyonu (ROP) ile kombinasyon halinde DA "click" reaksiyonları çeşitli mimarilere erişmek için kolay bir rotadır.

Bu tez çalışmasında kullanılan tetrazin aracılı IEDDA Click Reaksiyonu yüksek seçiciliği ve süper hızlı kinetiğe sahip olması nedeniyle önemli bir modifikasyon tekniğidir. Polimer zincirlerine tetrazin kısımlarının eklenmesi, çok aşamalı sentetik yollar ve fonksiyonel polimerik malzemelere yol açan bir polimerizasyon sonrası

adımı gerektirir. Bu tür yaklaşımlar, polimerin ve modifikasyonda kullanılacak olan molekülün ayrışmasıyla sonuçlanır.

Bir başka çalışma alanı olan ışıkla tetiklenmiş fonksiyonel kaplamalar, günümüz toplumunun enerji ve çevre ile ilgili sorunlarına yönelik malzemelerin başarılı, etkili ve verimli bir şekilde kullanılması için önemli bir teknoloji sunmaktadır. Fotokatalist kaplamalar geleneksel olarak fiziksel / kimyasal buhar biriktirme veya sol-gel ile ilgili teknikler yoluyla elde edilir.

Bu çalışma kapsamında IEDDAreaksiyonunun ışıkla tetiklenebilir versiyonu kullanılarak cam yüzeylerin modifikasyonu yapılmıştır. Bu reaksiyonda gerekli olan moleküllerden tetrazin molekülünün fotooksidasyon yoluyla elde edilmiştir. Bunun için iki farklı strateji uygulanmıştır; (1) dHTz fonksiyonel substratın (yani polimer) *trans*-5-hidroksisiklookten (TCO) ile modifiye edilmiş yüzeylere uygulanması ve (2) TCO-fonksiyonel substratın (yani protein) dHTz ile modifiye edilmiş yüzeylere uygulanması.

İlk stratejide, cam yüzeyler, sırasıyla 3-aminopropiltrietoksisilan (APTES) ile tipik bir silanizasyon işlemi yapıldı ve ardından TCO molekülleri ile modifiye edilmiştir. PNIPAAm RAFT polimerizasyonu ile sentezlendi ve polimerin karboksilik asit uç grubu amidasyon yoluyla dHTz türevi (PPA-dHTz) ile fonksiyonlandırılmıştır. Sonuç olarak, dHTz fonksiyonel uçlu PNIPAAm, dihidrotetrazinin tetrazine *in situ* fotokimyasal dönüşümü ve IEDDA click reaksiyonu ile TCO fonksiyonelleştirilmiş cam yüzeylere bağlanmıştır.

İkinci stratejide, TCO-fonksiyonel proteinler foto-IEDDA yoluyla dHTz ile fonksiyonlandırılmış cam yüzeylere bağlanmıştır. Her iki durumda da, yüzeyler su temas açısı ölçümleri (WCA), SEM ve FT-IR spektroskopi analizleri ile karakterize edilmiştir.

Burada kilit nokta dHTz'nin tetrazine dönüşümü esnasındaki oksidasyon basamağının fotokimyasal olarak gerçekleşmesi ve in situ oluşan tetrazin molekülünün klik/konjugasyon reaksiyonunu vermesini sağlamaktır. Bu çalışma ile ileri teknolojik uygulamalardaki malzeme yüzeyinin modifikasyonunu sağlayacak, hızlı, etkin, ışık ile tetiklenebilir tetrazin click reaksiyonuyla ilk defa cam yüzeyler modifiye edilmesini sağlayacak bir yöntem geliştirilmesi hedeflenmiştir.

Ayrıca bu yenilikçi yaklaşımı kullanarak protein-polimer konjugatları (PPC) hazırlanmıştır. Bu konjügatlar, reaksiyonun ortak molekülü olan TCO ile ışık altında oluşturulan tetrazin molekülünün ve polimer bağının yardımıyla ışığa bağlı bir IEDDA reaksiyonu gerçekleştirilerek hazırlanmıştır.

Bu amaçla, dHTz esas olarak siyano gruplarına sahip dipiridil öncüleri ile sentezlendi ve karboksilik asitle sonlanan PNIPAAm-COOH gibi terminal polimer grubuna bağlanmıştır. Öte yandan, proteinler (Concanavalin A ve sığır serum albümini, BSA), PBS ortamında TCO molekülü ile fonksiyonelleştirilmiştir.

Son olarak, dHTz fonksiyonel PNIPAAm ve TCO fonksiyonel proteinler ışığa bağlı IEDDA yoluyla konjüge edilmiştir. Fonksiyonlandırılmış cam yüzeylerin hazırlanmasında kullanılan, dHTz'nin tetrazine dönüşümü sırasında oksidasyon

aşamasının fotokimyasal klik / konjugasyon reaksiyonu ile gerçekleşmesini sağlayan strateji, PPC'nın hazırlanması için de kullanılmıştır. Hazırlanan konjugatlar kütle spektroskopisi (MALDI-TOF) ile analiz edilmiştir. Devam etmekte olan bu yaklaşım gelecekteki çalışmalarla iyileştirilebilir.

1. INTRODUCTION

Today, material science and chemistry play an important role in the working principle and technical knowledge (know-how) of many of the advanced technological applications, especially health and biomedical applications. Polymers are one step ahead of other materials in many applications due to their advantages. Currently, one of the most crucial issues of polymer science is to obtain functional polymeric materials in different topology and architecture by modifying the polymers. Therefore, many approaches have emerged in which polymerization techniques are combined with modification techniques. In the 2000s, polymer science and technology advanced one step further with the modification methods applied to the polymers in the 2000s [1], known as "Click" Chemistry[2], with efficiency, no side products and moderate conditions [3] Click chemistry describes bimolecular reactions in which effective and highly efficient covalent bonding occurs between two substrates or a substrate and a surface. Because it contains all the important standards of click chemistry, the copper (I) catalyzed 1,3-dipolar azide-alkyne cyclo-addition (CuAAC) reaction is frequently used in fields such as material science and drug development [4]. However, due to the toxic properties of the metallic catalyst in biological environments, systems with non-offensive characteristics against these environments have been developed. These approaches, known as bioortogonal reactions, are not affected by complex biochemical reactions in biological environments with hundreds of functional groups, nor do they show any negative effects on biochemical reactions [5]. In biological applications, these approaches are often referred to as bioortogonal conjugation [6]. Bioortogonal conjugation reactions are often used in *in vivo* studies such as cell labeling, cell imaging, tumor imaging and PET imaging techniques, as is common in *in vitro* studies such as protein labeling, protein modification and proteomics [7].

In this thesis, it is aimed to develop a photochemically induced click reaction based on tetrazine mediated high-speed IEDDA reactions. The IEDDA reaction shown in Figure 1.1 takes place quantitatively in a short time and has a short half-life of 10 seconds. The reaction takes place between the tetrazine molecule and *trans*-cyclooctene,

releases nitrogen gas into the medium, giving the coupling product. The R groups on the molecules can be polymer, protein, surfaces or small molecules to attach one to another one (Figure 1.2). This approach was shown in grafting glass surfaces and protein-polymer conjugates.

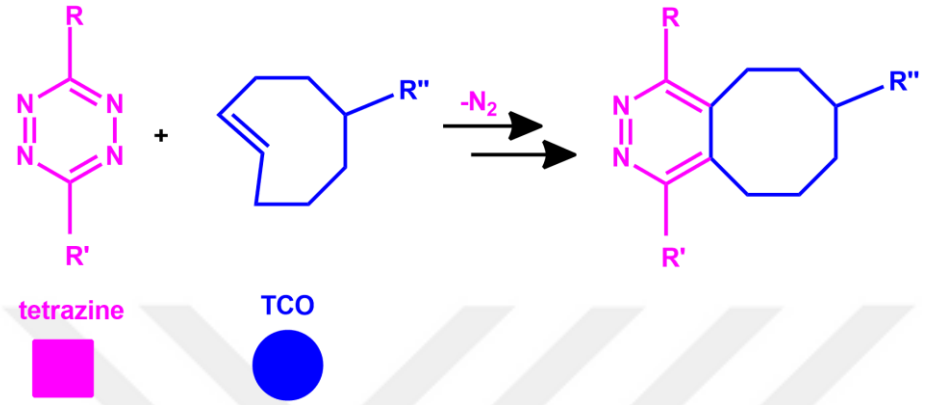


Figure 1.1 : Tetrazine *trans*-cyclooctene IEDDA reaction.

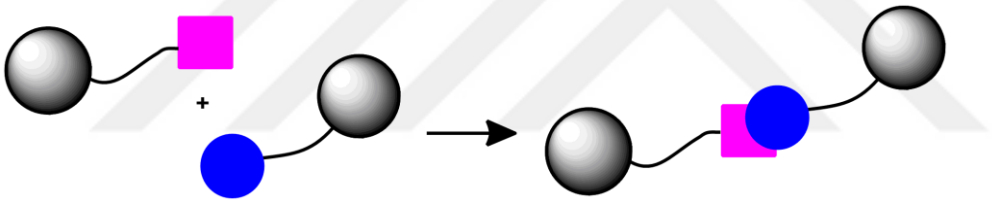


Figure 1.2 : Linking of two molecules with Diels-Alder Click Reaction.

Surface modifications have been widely employed for grafting functionalities to surfaces to acquire biological activity[8], anti-microbial or anti-fouling feature[9], biocompatibility[10], sensor molecules[11], chemical resistance or activity[12], additional hydrophobicity[13] or hydrophilicity[14], corrosion inhibition[15], stimuli response[16] and etc. There are two chemical modification approaches in which chemical bonds are formed between the surface and the substrate of interest yielding more durable and robust surfaces; “grafting from” and “grafting to”. Most of the “grafting to” strategies for fabrication of functional surfaces rely on click reactions. Light-induced click reactions provide temporal and spatial control in surface modification leading to tailor-made patterning[17].

Herein, a novel surface grafting approach based on photo-IEDDA click reaction has been developed. The approach depends on in situ formation of tetrazine molecule in the reaction medium and a subsequent IEDDA reaction of tetrazine moieties with *trans*-cyclooctene groups. Firstly, light-induced formation of a tetrazine derivative from a precursor, dihydrotetrazine (dHTz) molecule, in the presence of a photosensitizer, namely Methylene Blue under visible light irradiation (LED, 100 W) was evaluated. The conversion of dHTz to tetrazine was examplified on photo-induced oxidation of a model precursor, 6-(6-(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (PPA-dHTz), to corresponding tetrazine (6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine) which was monitored by NMR spectroscopy. For grafting glass surfaces, dHTz end functionalized poly(N-isopropylacrylamide) (PNIPAAm-dHTz) was prepared by RAFT polymerization, and post-polymerization with a dHTz molecule (PPA-dHTz). While, glass surfaces were decorated with TCO groups by functionalization with 3-aminopropyltriethoxysilane, succinic anhydride and *trans*-cyclooctenol (TCO) successively. Then, photo-IEDDA was performed between the dHTz end functionalized polymers and TCO-decorated glass surface in the presence of Methylene Blue and under visible light irradiation (LED, 100 W). The modified glass surfaces were evaluated with water contact angle measurement, scanning electron microscopy and FT-IR spectroscopy. To extend the applicability of the approach, grafting TCO-functionalized protein, namely Concanavalin A-TCO, to dHTz-modified glass surface was achieved as well. In this case, glass surface was functionalized with PPA-dHTz. In both cases, glass surfaces were grafted successfully with the polymer and the protein through the light-induced approach which would offer spatial and temporal control in functionalization surfaces.

Protein-polymer conjugates are complicated molecules, used in many major applications, and the development of these bioconjugates has occurred with advances in the fields of medicine, biotechnology, and nanotechnology. Furthermore, the synthesis of various unique materials exhibiting functional properties such as thermo response [18], exceptional stability and specificity were made possible by the development of new biomolecule-polymer conjugates containing protein, peptide or nucleic acid [19, 20]. In addition, bioconjugated therapeutic agents have been used as a selective drug delivery system for many treatment methods[21, 22]. One of the most rapidly developing areas of modern science is spatial and temporal control[23]

throughout chemical and biological processes [24], both for the preparation of protein-polymer conjugates and for controlled production [25, 26]. Another study of this thesisis preparation of PPC via light-induced version of IEDDA reaction[27] with high efficiency [28, 29]. For this purpose, dHTz is synthesized primarily with dipyridyl precursors having cyano groups and is attached to the terminal group of polymers such as carboxylic acid-terminated PNIPAAm-COOH. While, proteins (Con A and BSA) with TCO molecule were prepared in PBS medium. The polymer and the protein were conjugated in the presence of Methylene Blue under illumination of white light (LED, 100 W). The conjugates were analyzed with MALDI-TOF.

2. THEORETICAL PART

2.1 Brief Explanation of IEDDA Reactions

Copper-catalyzed azide-alkyne cycloaddition (CuAAC) as a conjugation reaction is of great interest due to its properties such as the reaction obtaining high efficiency and taking place under moderate conditions. However, due to the use of copper based catalyst with toxic effects, the need for extra functional groups and slow kinetics, there is increasing effort for different bioortogonal reactions. As a result, many bioortogonal reactions have been developed, such as succinimide-amine, Michael addition (thiol-olefin), Staudinger ligation (phosphine-azide), strained ring alkyn-azide and aminoxy-carbonyl reactions. However, IEDDA Click Reaction, which has been developed in recent years and is the fastest conjugation method known in the literature, has the potential to overcome the issued related to other click reactions [30]. Tetrazine reaction between alkene or alkyne dienophile, namely IEDDA Click Reaction, has emerged as an essential reaction as a bioorthogonal ligation called also tetrazine ligation recently [27] . (Figure 2.1)

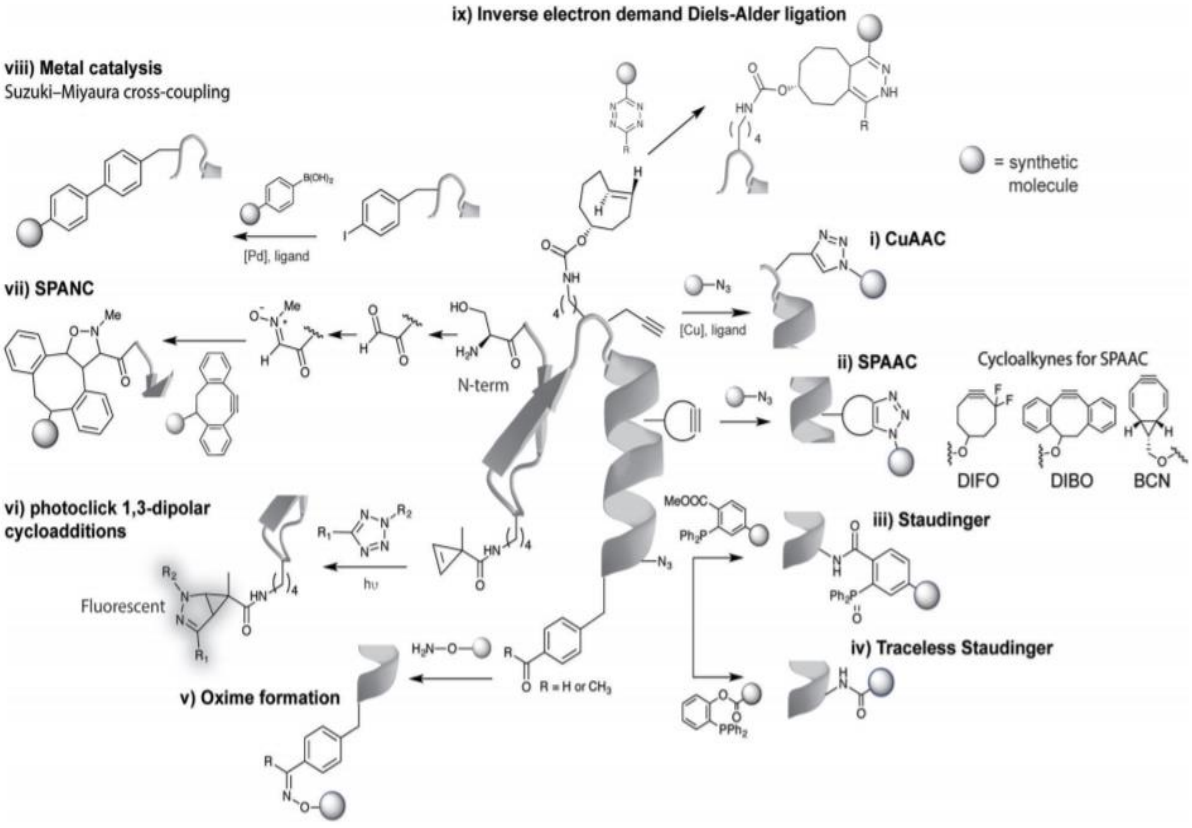


Figure 2.1: Examples of bioorthogonal reactions for protein modification [31].

(**i**) CuAAC, (**ii**) SPAAC; the strain-promoted [3+2] azide–alkyne cycloaddition, it has slower kinetics when compared to the **i**, (**iii**) Staudinger; produces an amide bond while azides produce a triazole linkage in **i** and **ii** reactions, (**iv**) traceless Staudinger; the general reaction is a mild method of reducing an azide to an amine, and triphenylphosphine or tributylphosphine are the most commonly used reactants. (**v**) hydrazones and oximes formation (**vi**) photoclick reactions (**vii**) strain-promoted alkyne-nitrone cycloaddition (SPANC) reactions (**viii**) transition metal catalysis and (**ix**) IEDDA reactions [31].

Based on the basic molecular orbital theory, there are two components in the classic DA reaction; the electron-rich diene and the electron-poor dienophile.(Figure 2.2)

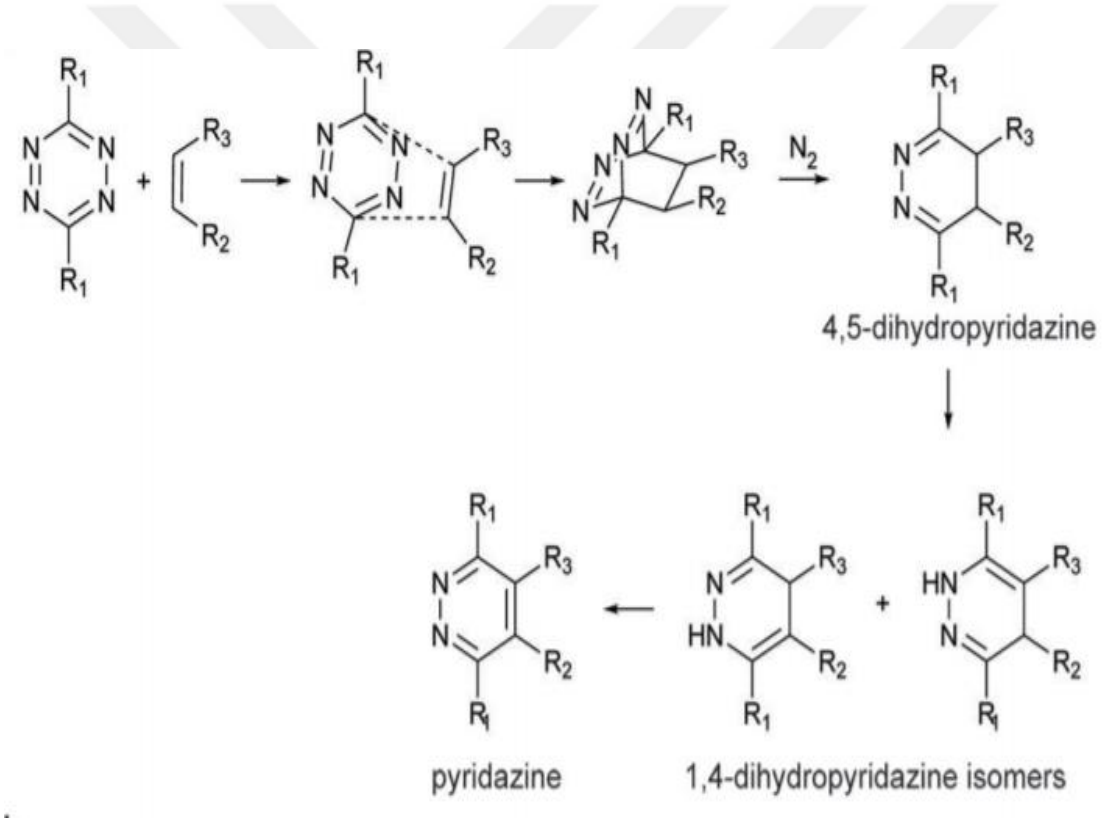


Figure 2.2 : Schematic illustration of the DA reaction of tetrazine [31].

Diene and dienophile have an equal electron density in dimerization reactions. In the IEDDA reaction, LUMOdiene and HOMOdienophile have higher energy levels compared to HOMOdiene and LUMOdienophile, which could interact and construct most effectively result in the most energetically optimal bonding with high kinetics as shown in Figure 2.3.

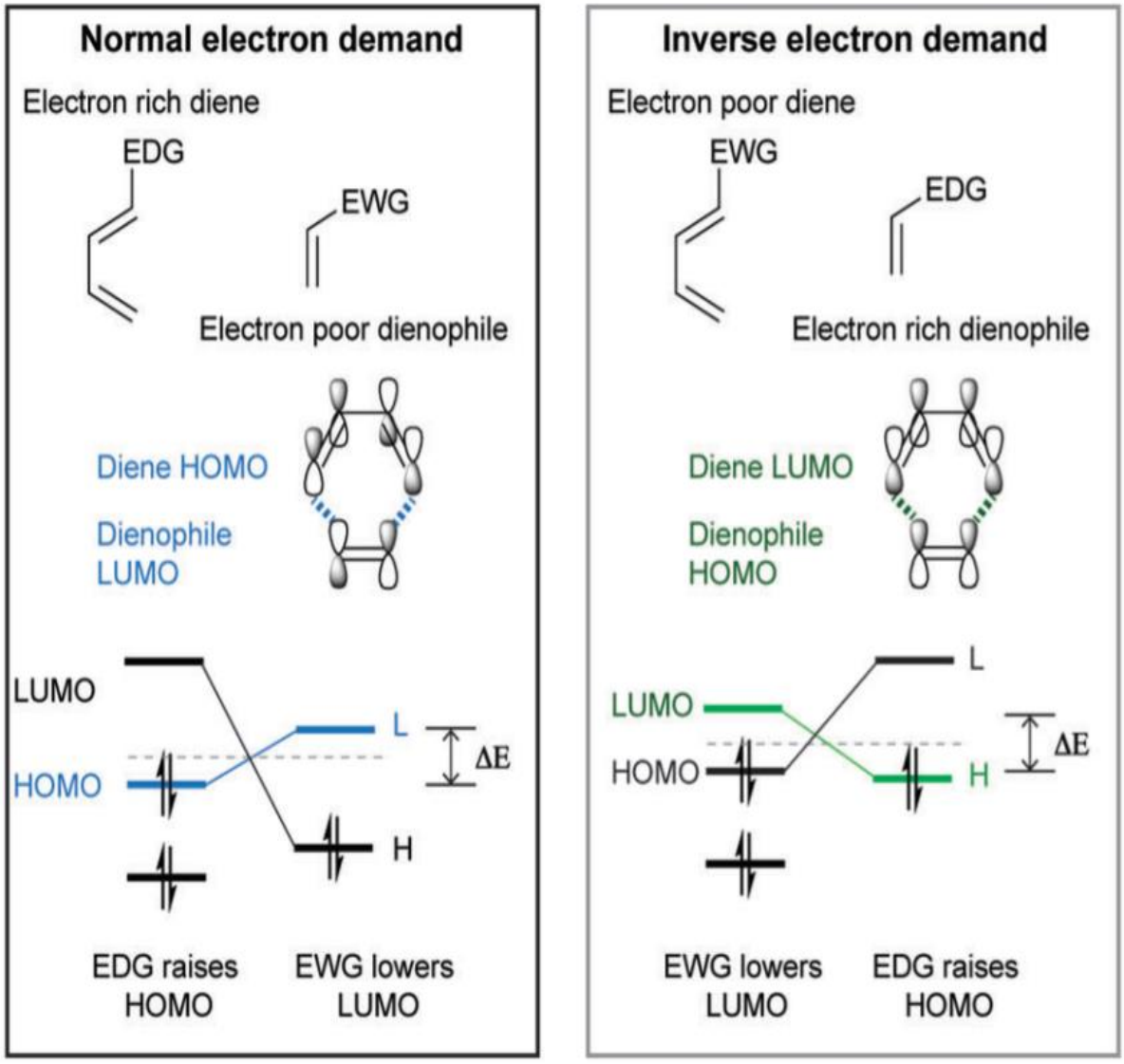


Figure 2.3 Comparison of Frontier molecular orbital DA and IEDDA.

EDG means electron-donating group and EWG; electron-withdrawing group. [31].

2.2 Tetrazine IEDDA Click Reaction

2.2.1 Tetrazine

Tetrazine is a compound containing four nitrogen atoms and a six-membered aromatic ring, formulated with $C_2H_2N_4$. The name Tetrazine is used to name derivatives of this compound(Figure 2.4) [32].

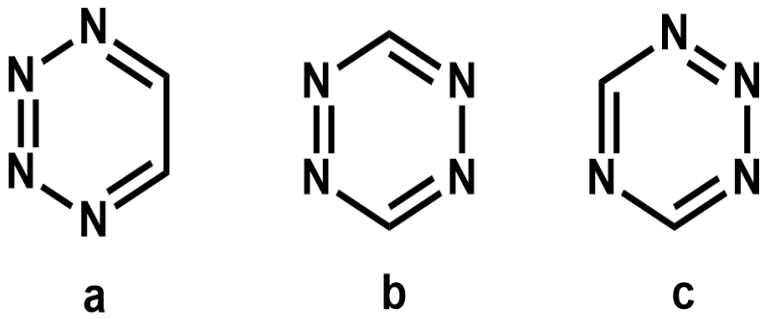


Figure 2.4: a) 1,2,3,4-(v)-tetrazines, b)1,2,4,5-(s)-tetrazines and c) 1,2,3,5-(as)tetrazines.

It could be said that there are three main substrates for tetrazine mediated bio-orthogonal reactions. In terms of reaction kinetics, the most preferred substrate molecule for tetrazine ligation is *trans*-cyclooctene (TCO)[33]. Norbornene is not preferred due to its slow kinetics although it is cost effective. The reason why cyclopropene is not preferred is that it is an unstable molecule although it has a fast reaction kinetics (Figure 2.5).

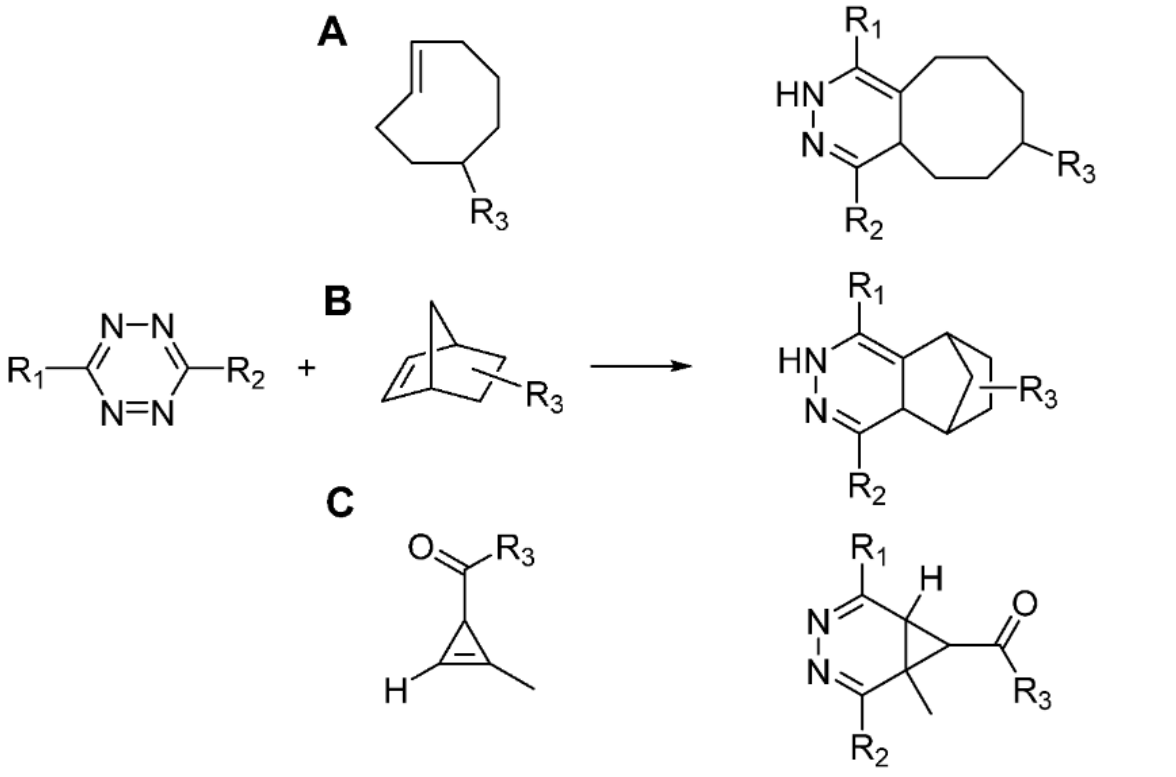


Figure 2.5 : A) TCO, B) norbornene, C) cyclopropene [34].

2.2.2 Dihydrotetrazines

Tetrazines precursors before oxidation are named as dihydrotetrazines, which are used with oxidizing agents in the synthesis of tetrazines. For example, 1,4-dihydro-s-tetrazine precursors are usually selected to synthesize S-tetrazines, which are then oxidized to give s-tetrazine products.Nitrous reagents (e.g HONO, $NaNO_2$ and isoamilnitrite) are generally used for oxidizing 1,4-dihydro-s-tetrazines to s-tetrazines. This method often continues with excellent efficiency. However, in some cases, medium yield has been reported and nitrogen reagents are not preferred for sensitive substrates [28].

Today, the preparation of s-tetrazines makes it necessary to improve ideal, credible and effective methods.

Amino-substituted di(pyridin-2-yl)-s-tetrazine is an example of s-tetrazine, which could not be procured by oxidation with nitrogen reagents as mentioned in Figure 2.6.

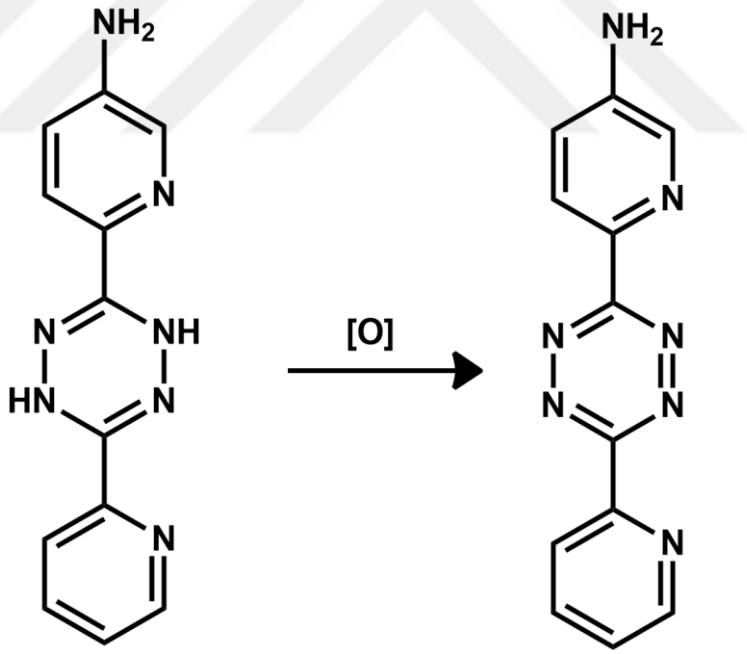


Figure 2.6 : Oxidation of amino-substituted di(pyridin-2-yl) -s-tetrazine.

PhI(OAc)2 has been noticed as a modest and effective oxidant that matters to oxidation of dihydrotetrazines for the synthesis of s-tetrazine derivatives (Figure 2.7).

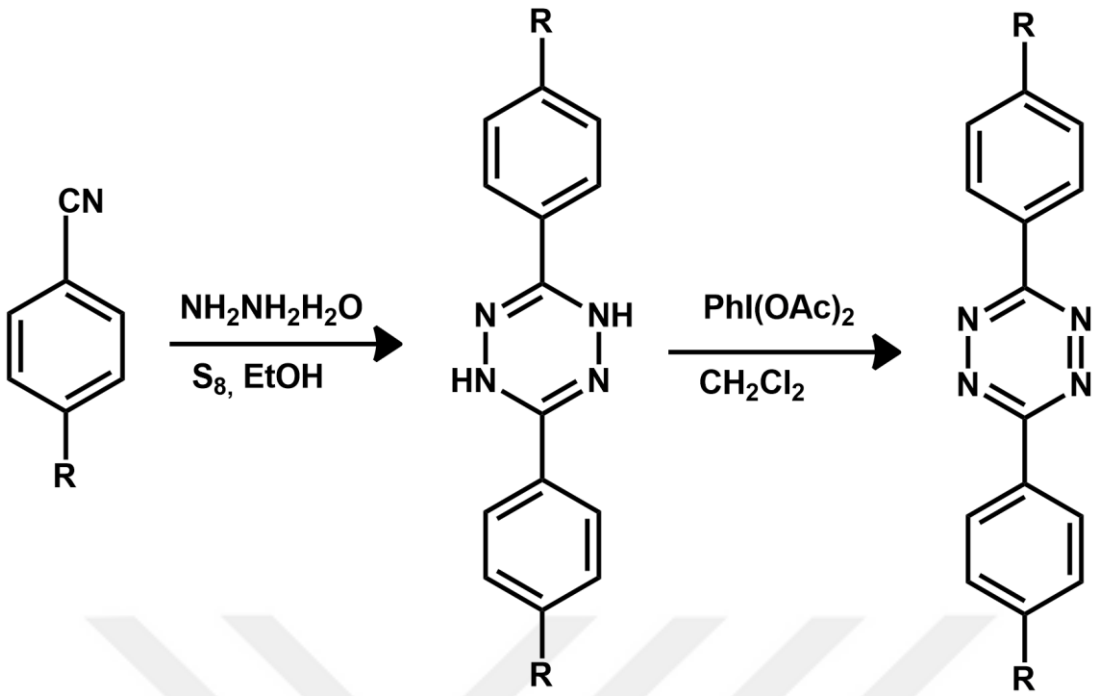


Figure 2.7 : General synthesis reactions of s-tetrazines.

2.2.3 Tetrazine ligation

Tetrazine chemistry has been widely employed in cell labeling, intracellular small molecule imaging, genetically target protein labeling, post synthetic DNA labeling, nano-particle-based clinical diagnostics, in vivo imaging are used because of its high speed, selectivity and biocompatibility [33, 35]

For instance, in an interesting study,[36] surface of one of two different living cells was functioned with tetrazine (Tz) and the other with *trans*-cycloocene (TCO), and these two cells were incubated in the same environment and connected to each other.

This bioortogonal click approach has been described as a cell adhesive due to its high efficiency and kinetics. (Table 2.1) [37]

Table 2.1 : Frequently used click reactions and reaction rates

Reaction	**Reagents**	**Rate [M^{-1} s^{-1}]**
CuAAC	Alkyne- azide	$10^{-2} - 10^{-4}$
Staudinger	Phosphine-azide	$2.2x10^{-3}$
Insufficient Electron AAC	Diflorocyclooktine-azide	$8x10^{-3}$
Strained ring AAC	biarylazacyclooctinone-azide	1
IEDDA	Tetrazine-norbornene	1-10
IEDDA	Tetrazine-cyclooctene	$3.8x10^{5}$

This effective chemistry has also been used in many valuable macromolecular engineering aprroaches such as surface modification[38], hydrogel synthesiss[39], polymer functioning [40].

As an ideal couple of IEDDA Reaction, TCO - Tz could be effective in biological treatments such as protein-protein conjugations, which has low concetration. This ligation chemistry is based on IEDDA reaction between a *trans*-cyclooctene and tetrazine reaction pair and forms a dihydropyridazine bond (Figure 2.8).[41]

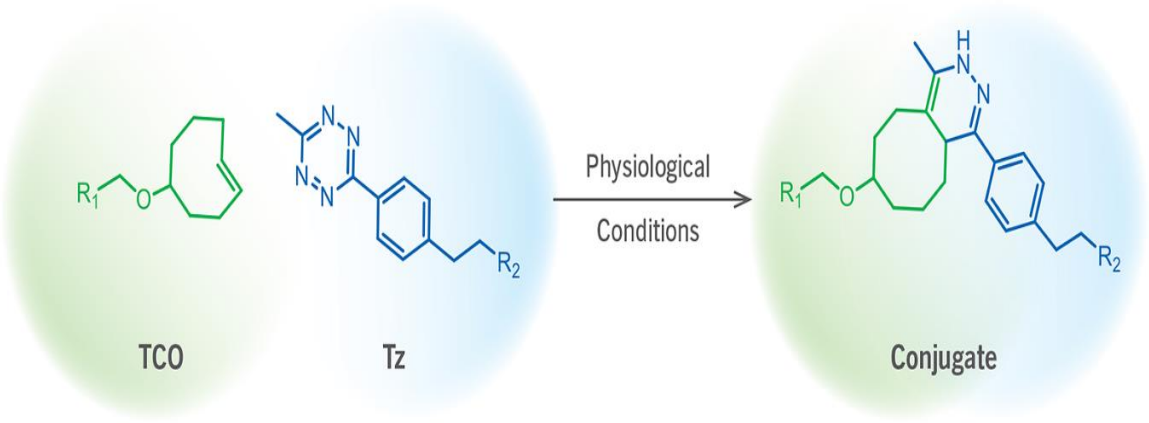


Figure 2.8 : Schematic illustration of a TCO–Tz ligation reaction [41].

Reactivity is an absolute prerequisite for applications performed under highly dilute conditions such as protein-protein conjugations. For bio-orthogonal reactant pairs, the second order rate constant can be defined as reactivity.

The higher the second order rate constant, the more efficient the conjugation is at appropriate time scales, low concentrations, near neutral pH, and without use a large amount of both biomolecules. This chemoselective TCO-Tz ligation pair has a unique ultra-fast kinetics (> 800 $M^{-1}s^{-1}$) compared to any other bio-ligation pair. The relationship between second order velocity constants ($M^{-1}s^{-1}$) for bio-orthogonal reactants at 10 μM and the percentage of conjugated yield over time is shown in Figure 2.9. [41]

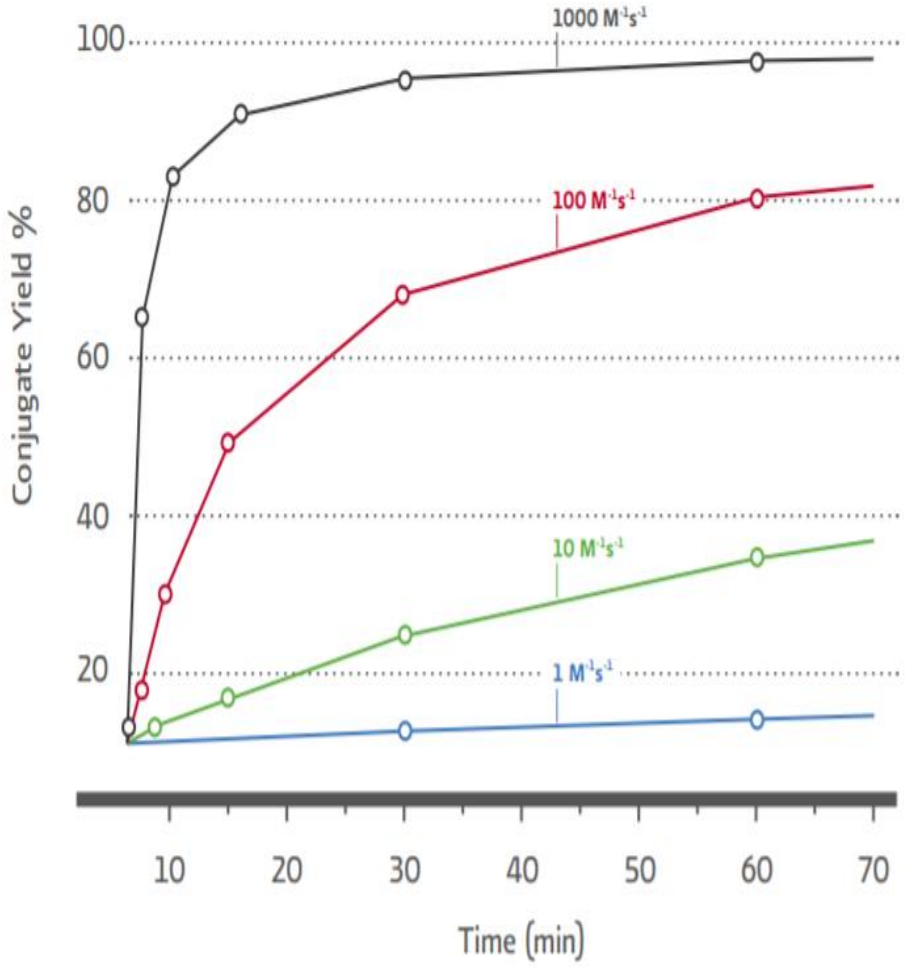


Figure 2.9 : Simulation of second order reactions of TCO-Tz ligation at 10 μM [41].

2.3 Light-Induced Click Reaction

Light-induced click reactions, in which the reaction is triggered upon absorption of light by one of the component (usually a photosensitizer), circumvent the issue related to uncontrolled initiation of conventional click reactions. Therefore, light-induced reactions offer spatial and temporal control over the corresponding reactions. Such remotely inducible reactions excellently combine advantageous features of conventional click reactions with the advantages of photochemical processes.[24]

The light-induced version of IEDDA(photo-IEDDA) has been also developed recently due to its advantageous features although there are limited number of examples.

Photo-IEDDA reactions that are preferred as an inspiring method in this study have applied as the main method to develop biological orthogonal reactions due to its control mechanism and excellent kinetics (Figure 2.10).

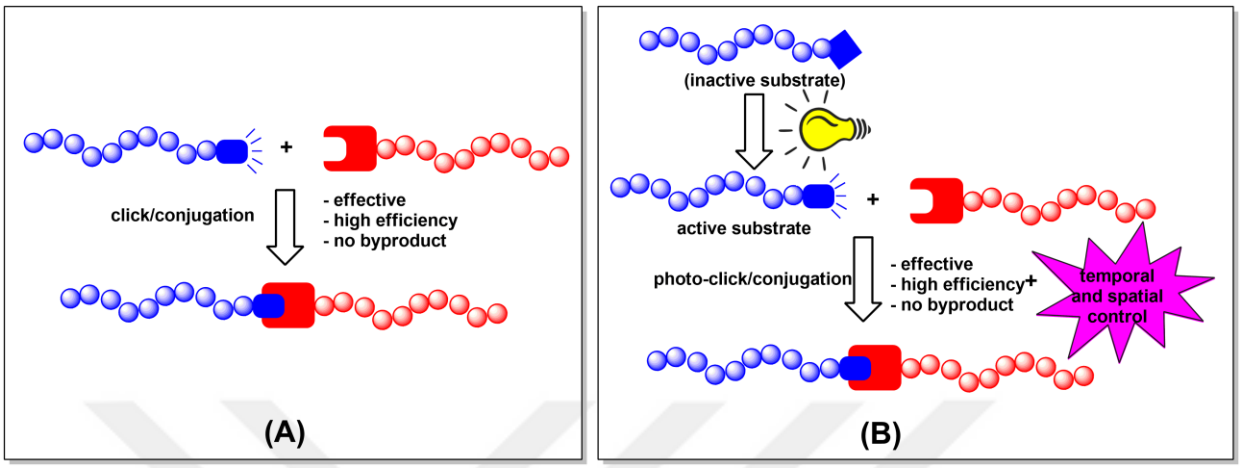


Figure 2.10 : Classic Click /Conjugation Reaction (*A*) and Light-Induced Click /Conjugation Reaction (*B*).

2.3.1 Tetrazine mediated light-induced IEDDA reaction

Basically, there are two distinct approaches of photo-induced tetrazine ligation; (1) photoactivation of a caged-substrate and (2) in situ formation of tetrazine. In the first strategy, cyclopropenone-caged bicyclononyne [42] or caged cyclopropene[43] was photochemically activated in situ and reacted with tetrazine. The caged olefin substrates are in situ transformed to active olefin substrates that immediately give photo-IEDDA reaction with tetrazine molecules present in the medium. In the second approach, tetrazine precursors are photocatalytic activated in situ in the presence of an active substrate.

The first example of this photo-IEDDA click reaction was reported by Fox and co-workers[44]. In this approach, tetrazine was formed in situ by a photo-chemical oxidation of corresponding precursor, dihydrotetrazine, and subsequently reacted with a proper substrate, namely a *trans*-cyclooctene derivative.

In a typical synthesis of tetrazine molecules, two nitrile molecules are combined with each other in the presence of hydrazine and a catalyst (sulphur or a Lewis acid) yielding dihydrotetrazine rings. In the final step, dihydrotetrazines are chemically oxidized to corresponding tetrazines using an oxidant (usually sodium nitrite) in acidic medium.[45] Unlike, dihydrotetrazine is photochemically oxidized in the presence of

a photosensitizer (i.e. Methylene Blue) in photo-IEDDA. Fox and others applied this chemistry in fabrication of biologically active fibers through interfacial polymerization.

In another study, Forsythe and co-workers employed the similar chemistry (norbornene was used as substrate instead of *trans*-cyclooctene) in preparation of hydrogels.[29] According to this report, the preferred tetrazine - norbornene (Tz - Nb) additions have been used in the preparation of biological orthogonal cross-linked hydrogels. The dHTz groups placed in the polymer chain oxidized under long wavelength visible light, making it possible to add Tz-Nb to the polymer. Here, red light was applied under physiological conditions for polymer conjugation by catalytic activation of IEDDA [29].

Another important point is that for most redox pairs, dHTz is easily oxidized in air or Tz is very reactive to water and other nucleophiles. This reference article shows that the dipyridyl dHTz / Tz pair has good stability in both cases [27]. For this reason, precursors with bipridyl group were used for synthesis of the dHTz molecules in this study In addition, this study proved that oxygen is not an oxidizing agent for dipyridyl dHTz in photocatalytic conditions. The photooxidation mechanism is largely based on electron transfer and studies on this subject are ongoing.

2.4 RAFT Polymerization

Reversible Addition - Fragmentation Chain Transfer, RAFT polymerization, which is defined as the living radical polymerization type, was discovered in 1998. This method defined as the traditional free radical polymerization that a monomer in the presence of a proper chain transfer agent (RAFT agent or CTA), which could give novel opportunities to construct complex macromolecules [46]. The use of a suitable RAFT agent enables the synthesis of polymers with low polydispersity index (PDI) and high functionality [47, 48]. Most commonly used RAFT agents are given in Figure 2.11.

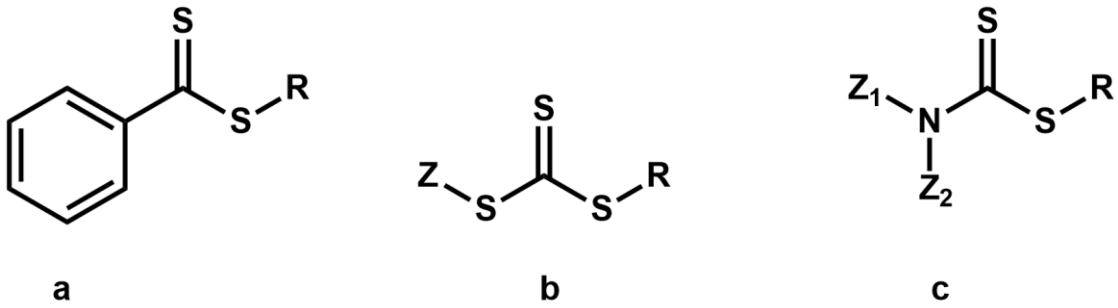


Figure 2.11 : a) dithiobenzoates b) trithiocarbonates c) dithiocarbamates.

Dithiobenzoates; for RAFT polymerization of methacrylates and methacrylamides, they are considered to the preferable control over molecular weight and end-group stability.

Trithiocarbonates; although they are less forceful than other RAFT CTAs, they are easily synthesized than other agents and effective at controlling the polymerization for styrene and methacrylate monomers.

Dithiocarbamates; allow controlled polymerization as an agent and supply an alternative to obtain low-dispersity block co-polymer.

The CTA typically has a thiocarbonylthio group (S = C-S) with R and Z substituents that affect reaction kinetics and the degree of morphologic control. General RAFT agent structure is named as follows according to Z groups; tritiocarbonate Z = SR, dithioester Z = alkyl or aryl, dithiocarbamate Z = NR_2, xanthate Z = O-alkyl, R = alkyl or H (the structures of xanthates are not given). Polymerization would be realized using versatile methods, which as named classic, thermal, redox or photo-induced polymerization.

The selection of the appropriate RAFT reagent is the factor that will largely determine the success of the experiment for special reactants as shown in Figure 2.12 and Figure 2.13, which are only given progression steps of polymerization [49].

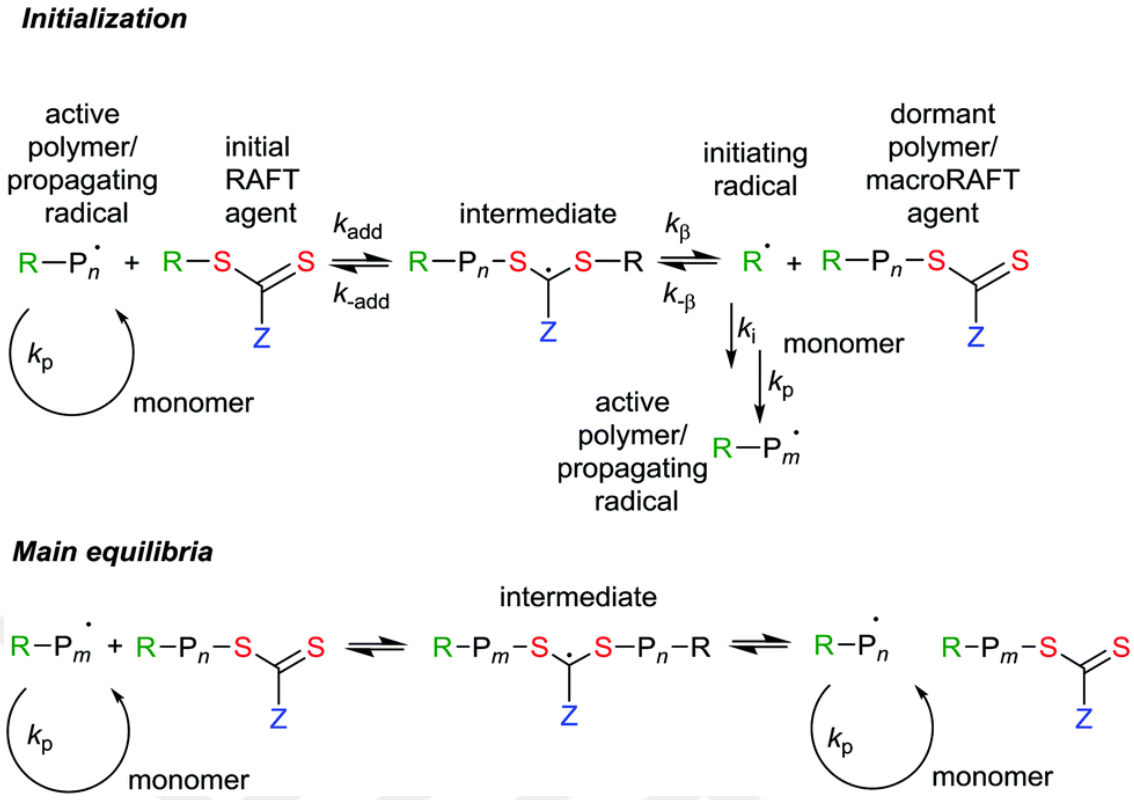


Figure 2.12 : Mechanism of RAFT polymerization[48, 49].

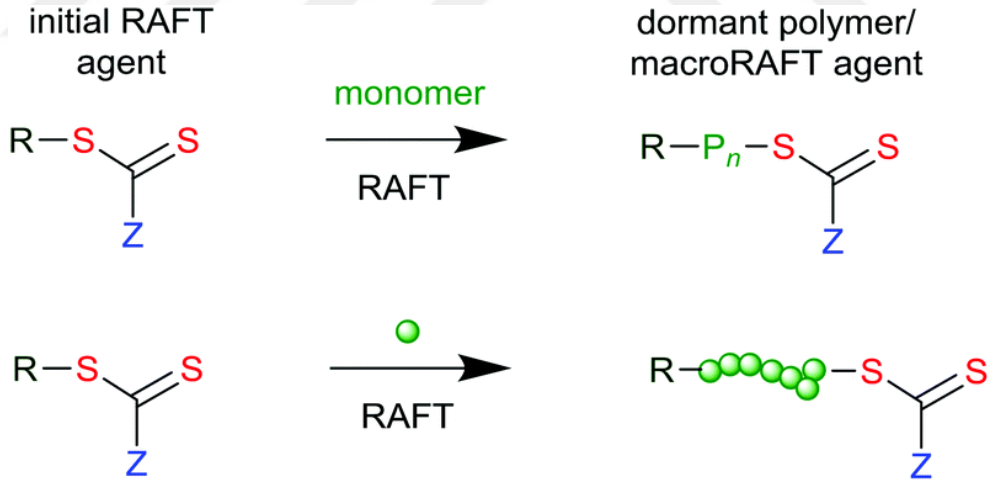


Figure 2.13 : Two different models of the overall RAFT process[49].

Monomer's reactivity in radical polymerization is classified as two main groups that "less active" and "more active" monomers. N-vinylpyrrolidone and N-vinylcaprolactam can be given as examples of less active monomers, which have a double bond that adjacent to one or more oxygen, nitrogen, halogen or sulfur atom. On the other hand, styrenes and vinyl aromatics, methyl methacrylate and acrylonitrile

are known as more active monomers, which contain a double-bound that conjugated with an aromatic ring a carbonyl or a nitrile respectively[49].

2.4.1 PNIPAAm synthesis via RAFT polymerization

Poly(*N*-isopropylacrylamide) is sensitive to heat polymer that was first synthesized in the mid-twentieth century and has been abbreviated in various ways, [50] but in this study we preferred to use as PNIPAAm. It could be produced from commercially available N-isopropylacrylamide (NIPAAm) and synthesized by free radical polymerization, which could be defined as a polymerization method in which a polymer is formed by adding free radical building blocks formed by the initiator molecules in succession [51]. Also, PNIPAAm is easily functionalized to be convenient in many diverse applications[52] according to the desired feature [53].

When crosslinked with *N*,*N'*-methylene-bis-acrylamide or *N*,*N*-cystamine-bis-acrylamide, it builds a three-D hydrogel (Figure 2.14). Transforming swollen hydrated state to a shrunken anhydrous state reversibly when heated in water above 32°C is the most critical characteristic known as lower critical solution temperature (LCST) phase transition. This feature of PNIPAAm, which discharging of the liquid content at a temperature close to the human body [54], allows it to be used in tissue engineering and controlled drug delivery systems, and studies on these issues are still ongoing [55].

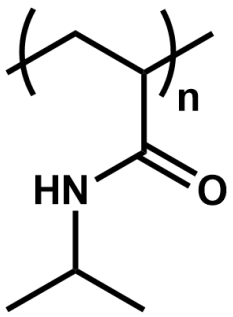


Figure 2.14 : The structure of PNIPAAm.

In terms of imitating hydrophilicity of three-D global patterns of natural proteins, linear and star homopolymers of N, N'-dimethylacrylamide were synthesized by RAFT polymerization, which could be given an application of PNIPAAm using as a copolymer. It was then used as macro chain transfer agents to carry out block copolymerization with NIPAAm. It was noticed that polymers combine as

monodisperse aggregates when heated above the LCST of PNIPAAm that in an aqueous pH 4 buffer solution. The aggregation temperature of the polymers and the size of the aggregates depend on the NIPAAm block size and the structure of the polymer [56].

In a current literature example, although an ordered thiol-ene and thiol-yne reaction has not been used before, consecutive thiol-ene / thiol-ene and thiol-ene / thiol-yne reactions have been used as an easy technique to modify of end groups in the NIPAAm homopolymer. LCST have been determined using a combination of optical measurements and dynamic light scattering, and LCST has been shown to range between 26-35°C degrees related to the end group of polymer [57].

2.4.2 Modification of RAFT-synthesized polymers

Designing of end-functionalized polymers are generally practiced by chain growth polymerization using the aforementioned techniques. Firstly, a functional polymerization initiator that has the desired functionality is synthesized in the pre-polymerization modification method. This approach is explained as a high degree of α-functionalization if the polymerization conditions are optimal. Due to required to the synthesis of a different initiator for every new a targeted functionalized polymer, this method has many drawbacks, which means over time consuming process and requiring a few steps for synthesis of precursors. Secondly, the most difficult part of post-polymerization, which is a reaction between polymer chain and a molecule of interest having a functional group, is that the coupling reaction must be impactful to meet for the polymer chain end that has the steric barrier [58].

RAFT is the most versatile controlled radical polymerization technique, as it tolerates a wide range of reaction parameters and enables control on a wide variety of monomers using different classes of CTAs. Recently, studies have been focused on the use of functional end group instead of removing sulfur-containing groups for finding solutions to the activity and degradation problems compared to early studies [59, 60]

Removing the end group of the polymers and then the ability to be functionalized is so essential and can be applied in many areas. According to the results obtained from the current studies, final group modifications of the polymers, which are synthesized by RAFT polymerization, could be possible. With all aforementioned advantages, RAFT is a superior polymerization route for the synthesis of polymers with functional end

groups, which are applied to various applications.We can divide the end group modifications into two main groups, α and ω (Figure 2.15)[61].

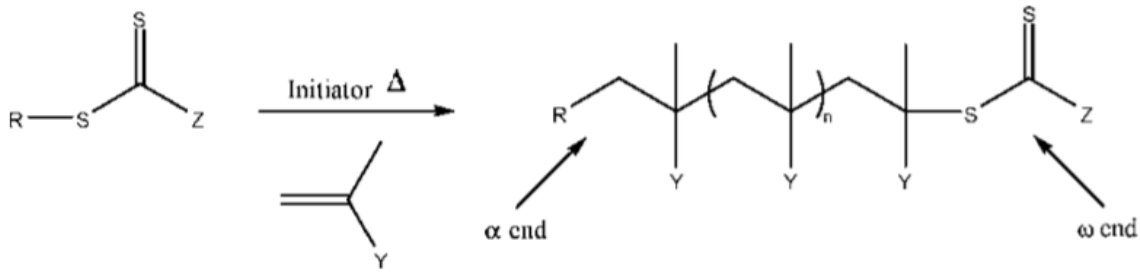


Figure 2.15 : Representation of the α and ω -ends of the polymer obtained by RAFT polymerization[61].

Alternatively, ATRP, which is another controlled polymerization method that could be preferred for controlling polymer's α-end group. By means of this mechanism, functional polymers are obtained by including the functionality of the initiator in the end group of the polymer [62].

2.4.2.1 ω- end modification

A new perspective of RAFT synthesized polymers for ensuring ω-end functionality of the polymer after polymerization is the presence of a thiocarbonate, which can be used in the end group of the polymer.

The thiocarbonyl group at the ω-end, which is a group with an electron deficiency, can be considered as a dienophile for hetero-DA reaction. (Figure 2.16)[63].

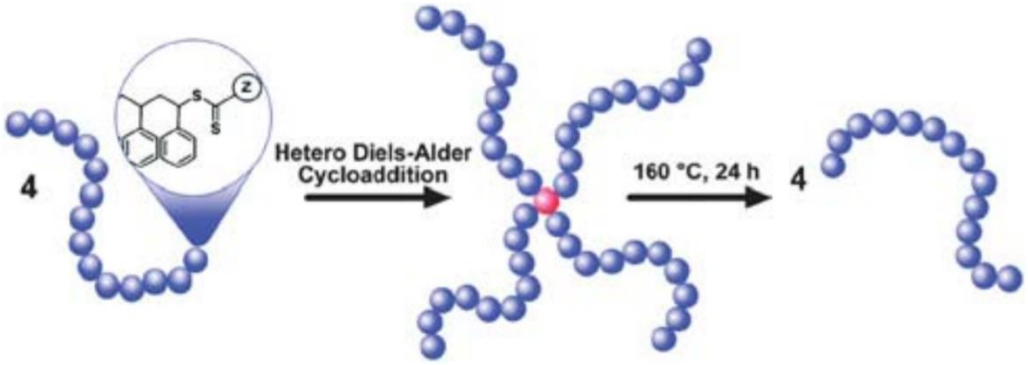


Figure 2.16 : Polystyrene stars that are synthesized with RAFT and hetero-DA combination [63].

Recent studies have proved that in the synthesis of complex macromolecular architectures such as stars, which are also important data for click chemistry, hetero-DA cycloadditions can be used again with high reactivity and efficiency.

2.4.2.2 α- end modification

In a recent article, for the first time, *in situ* synthesis of RAFT agent in the course of RAFT polymerization, based on the use of a carbonyl-azide precursor that reorganizes polymers of acrylate, acrylamide, methacrylate and styrene derivatives to an isocyanate [64]. It has also been proven that this RAFT agent is extremely active in preparing α-end functionalized polymers with as known a one-pot RAFT polymerization / "click" alcohol-isocyanate coupling approach, which presents new perspectives in the search for fast and simple synthesis way for targeted polymers. (Figure 2.17).

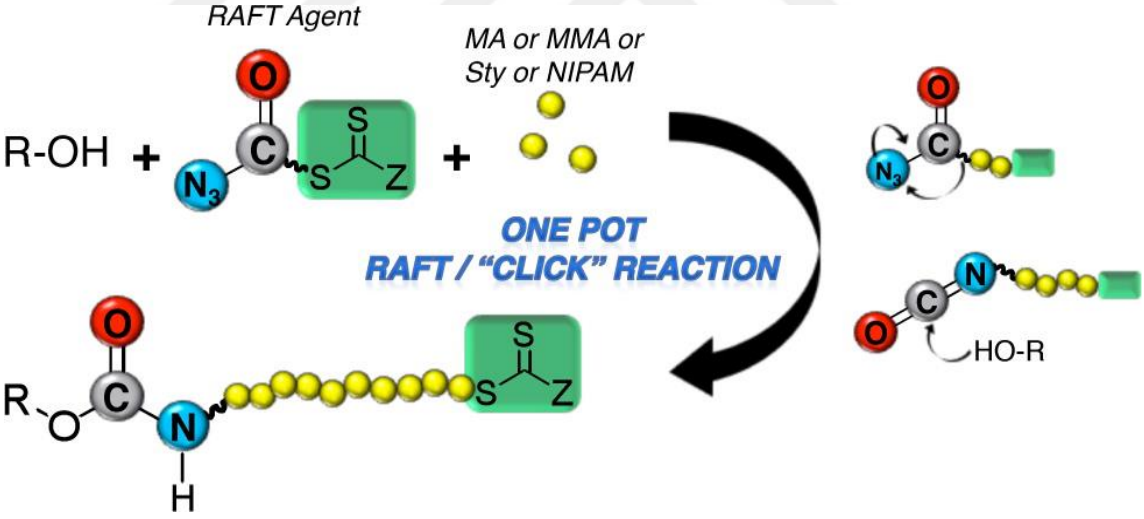


Figure 2.17 : Efficient synthesis of α-end functionalized polymers by RAFT polymerization[64].

The advantages of this strategy can be explained as follows; carbonyl-azide CTA is easily prepared in good yield and stored at room temperature without any damage, and a wide variety of monomers can be polymerized (e.g acrylates, acrylamides and styrenes) to originate well-defined a-isocyanate end functional polymers.

RAFT polymerization was used as a main tool in this study and can be expanded to other types of polymerization, radical- or non-radical-based, and reacted with different functional groups such as amines or thiols.

2.5 Surface Modification

Surface modification is defined as altering of morphological features that roughness, hydrophilicity, surface charge/ energy, biocompatibility and reactivity by a wide variety of methods, which is occurred basically in material's nature. Although surface modification is possible for specific liquids, the modification is usually done on solid materials [65].

2.5.1 Glass surface modification

Glass, which can be used in many areas, requires modifications in many cases. Changing the chemical composition of the glass or the surface of a glass product could be an effective way to enable it to reach tailor-made patterning [66]. The glass surface, also called solid glass, appears to be a sudden change between the air surrounding it. When the glass surface is examined more closely, different transition zones appear between the glass and the environment. Looking at the core of the soda-lime glass, it could be seen a silicon dioxide pattern that contains calcium and sodium ions are engrafted among them(Figure 2.18) [67].

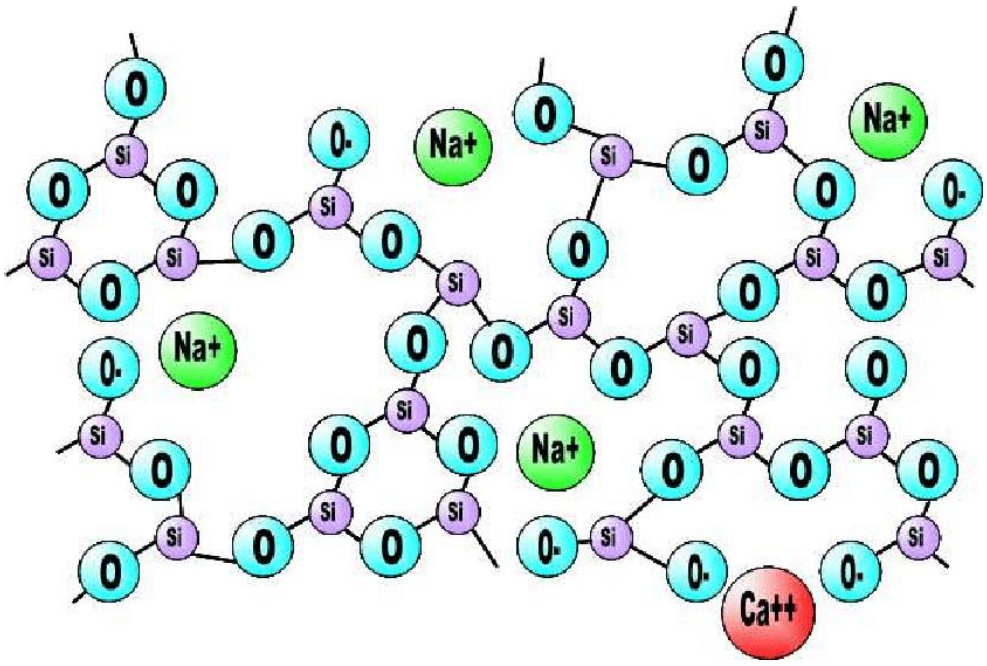


Figure 2.18 : Chemical structrure of a soda lime glass[66].

By nature of glass, water can cause some breakage on the silicon dioxide network connections and spread even more from the surface, and cause micro-cracks on the glass surface as shown in Figure 2.19.

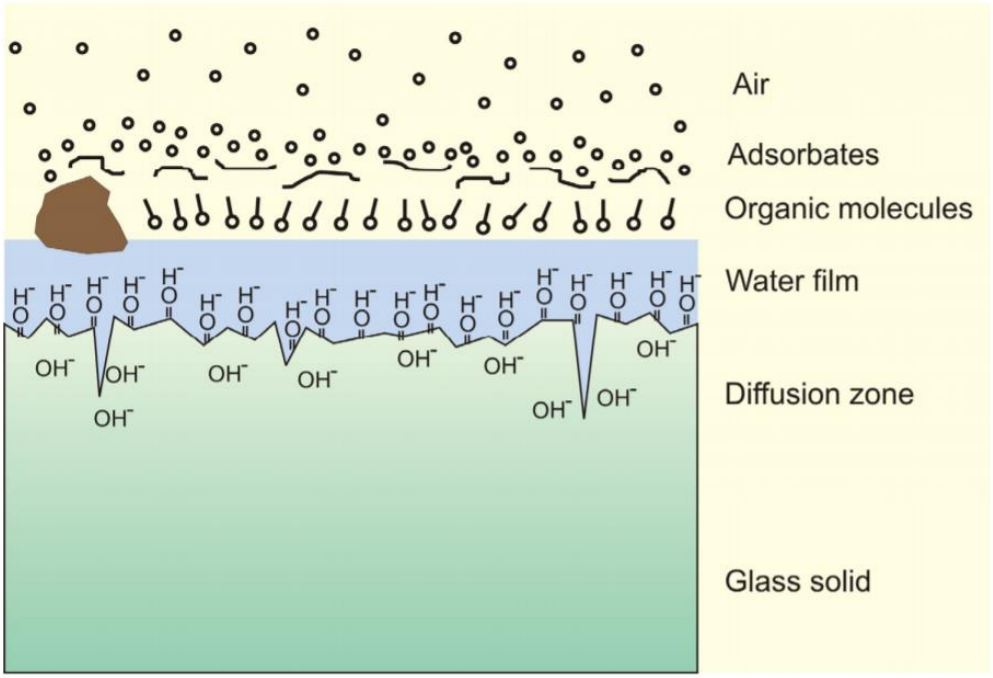


Figure 2.19 : Schematically glass surface before coating[66].

Changing the properties of a glass surface is generally possible in three ways. The first is to create a new surface by removing the material from the original surface, the second is to modify the original surface without creating a new surface, and the other is to create a new surface by adding extra materials.

A variety of methods are available for surface modification, which will be described briefly here [66].

The original surface cleaning from undesired molecules

Mechanical (grinding and polishing) and chemical (etching) route modifications can be made.

Modification of existing glass surface

Diffusion (glass staining or chemical toughening) route modification can be made.

Coating of the original surface

Plasma (ion coating), gas phase (spray/physical vapour deposition) or liquid/solid phase (coatings/sol-gel) can be given as examples of that method.

2.5.2 Recent examples of glass surface modification

Glass modification applications are common in the automotive industry, especially coating technologies are the most used methods for modifying automobile glass. For example, ultraviolet (UV) light protective glasses which are recently investigated to protect the driver's skin and hydrophobic thin rainy days film coatings, which is used to protect the mirrors' visibility can be given [68].

Due to the increasing demand of the consumer electronics industry, the laser processing technology of glass has made significant progress recently. Various methods have been improved from glass separation to surface modification. Flexible screens and ultra-thin devices are examples of daily use of glass surface modification[69].

In addition, glass surface modifications have been employed in a wide range of biomedical applications. For example, microarray technologies have gained great attention lately, as they are used as potential tools for high-efficiency analysis of biomolecular processes. In a recent biomedical study, a one-step acid-mediated method for modifying glass surfaces with N-hydroxysuccinimide esters was developed and applied to microarray studies [70].

Another example is bioactive glasses, which assist the repair process in living organisms, have morphology in nanoscale. In recent years, bone regeneration attempts have been tried and bone mimicking tissues have become possible with genetically modified stem cells with original bioactive glasses via delivery systems [71].

2.6 Protein-Polymer Conjugates

Bioconjugates with synthetic polymers are an effective and innovative way to add new value and unique properties to biomolecules. Polymer boconjugation aspect was originally developed by biochemists and has been studied only for biomedical applications, but today these macromolecules have increased in importance and have been used in many fields of material science. While various procedures exist for the preparation of such bioconjugates, it involves the preparation of functional polymer for binding to the biomolecule or the change of terminal group on polymer prepared by different polymerization methods for following click reactions [72, 73].

An example of conjugated nanoparticles; conjugates PNIPAAm / streptavidin (a protein in the tetramer structure obtained from Streptomyces bacteria) can be given [74].

Another use of PPC is the design of drug precursors. For example; when drugs combine with amino acids, they form an amide linkage and chemically conjugate, also amino acid prodrugs can be designed to have very good chemical stability and turn into parent drugs [75].

3. EXPERIMENTAL PART

3.1 Materials

3.1.1 Chemicals

4-(Aminomethyl)benzonitrile hydrochloride (Sigma-Aldrich, 97%), triethylamine (Merck), di-tert-butyl dicarbonate (Merck), sodium bicarbonate (Merck, 99.7%), magnesium sulfate (anhydrous, Sigma-Aldrich, 99.5%), acetonitrile (Merck), succinic anhydride (Sigma-Aldrich), triethylamine (Sigma-Aldrich), N-hydroxysuccinimide (NHS) (Aldrich), 1,3-dicyclohexylcarbodiimide (DCC) (Aldrich), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) (Sigma), 4-(dimethylamino)pyridine (DMAP) (Sigma, >98%), zinc trifluoro methane sulfonate (Sigma-Aldrich, 98%), hydrazine monohydrate (Sigma-Aldrich, 98%), sodium nitrite (Merck), cis-5-hydroxy-1-cyclooctene (Carbosynth), methyl benzoate (Sigma-Aldrich, 99%), chloroform (Sigma-Aldrich ≥99.5%), N,N-dimethylformamide (DMF) (Merck,99.8%), HCl (Merck), diethyl ether (Merck), silver nitrate (Sigma-Aldrich, ≥99%) were used as purchased. Con A from Canavalia ensiformis (jack bean) and BSA were purchased from Sigma-Aldrich and stored +4 ºC until use. Tetrahydrofuran (THF, Merck) was dried by refluxing over metallic sodium. Dichloromethane (DCM, Merck) was dried by refluxing over P2O5. Succinimidyl ester derivative of TCO (TCO-NHS)[76] was synthesized via brief synthetic route.

2-(((butylthio)carbonothioyl)thio)butanoic acid (CTA-COOH) was synthesized also following a literature procedure[77].

3.2 Characterization

3.2.1 Nuclear magnetic resonance spectroscopy (NMR)

All ^{1}H-NMR analyses were conducted on an Agilent NMR System VNMRS 500 spectrometer at room temperature in $CDCl_3$ or DMSO-d_6 with $Si(CH_3)_4$ as an internal standard.

3.2.2 Infrared spectrophotometer (FT-IR)

FTIR spectra were recorded on a Perkin-Elmer FTIR Spectrum One spectrometer.

3.2.3 Gel permeation chromatography (GPC)

A Tosoh EcoSEC dual detection (RI and UV) GPC system integrated with a Wyatt Technologies Dawn Heleos-II multi-angle light scattering (MALS) detector and a Wyatt Technologies DynaPro NanoStar dynamic light scattering (DLS) detector was used for molecular mass measurements. The eluent was DMF at a flow rate of 0.5 mL/min at 45°C. The GPC system contains Tosoh TSKgel G5000HHR column (7.8 x 300 mm), one Tosoh TSKgel G3000HHR column (7.8 x 300 mm), one Tosoh TSKgel SuperH-RC reference column for EcoSEC and one Tosoh TSKgel HHR-H guard column (6 x 40 mm). For data analysis Astra 7.1.2 software package was used.

3.2.4 Scanning electron microscope (SEM)

The morphology of glass surfaces coated with platinum was assessed using an ultra-high resolution scanning electron microscope (SEM; JEOL 7500F).

3.2.5 Water contact angle

WCA were anaylsed using a NanoLinker Contact Angle Measurement System (CA-500A) and a Hamilton microsyringe with a 22-gauge needle and sessile drop mode. A 5 μL water drop was added to glass surfaces, and the image of the water droplet was recorded after 10 seconds when the drop touched the sample surface.

3.2.6 Matrix-assisted laser desorption/ionization spectrometry (MALDI-TOF)

MALDI-TOF was analysed using LT MALDI-TOF MS(530 x 680 x 1093 mm (20.9“ x 26.8“ x 43“), temperature range: 16- 33°C (61-91°F) and operating humidity: 20-75% non-condensing at 33ºC (91°F), which is the compact microflex system and linear-mode.

3.3 Synthesis

3.3.1 Synthesis of poly (*N*-isopropylacrylamide) (PNIPAAm-COOH)

NIPAAm (660 mg, 0,0058 mol, 30 eq.) was dissolved with 2 mL of 1,4-dioxane and CTA(49.6 mg, 0.196 mmol 1 eq.) added after dissolved in 2 mL 1,4-dioxane, then AIBN (4, 8 mg, 0.029 mmol, 0.15 eq.) added in reaction mixture. The tightly sealed Schlenk tube was freeze-vacuum-thawed three times to remove gases dissolved in solution. The tube was saturated with nitrogen gas, then the reaction solution was

stirred continuously at 80°C for 6 hours. After that, the cooled contents were diluted with THF and the polymer was precipitated twice in diethyl ether. The filtered white polymer was washed extensively with diethylether. The impurity-free product had a gravimetric monomer conversion rate of 64.5% and a theoretical molecular weight of 2190 g / mol.

Characterizations of the polymers were carried out by using GPC and ^{1}H NMR.

3.3.2 Synthesis of 6-(6-(pyridin-2-yl) -1,4-dihydro-1,2,4,5-tetrazin-3-yl) pyridin-3-amine (PPA-dHTz)

Dipridyl compounds having cyano groups were used as starting materials to prevent instability issue of the precursor of amine-functional tetrazine [27]. Briefly, 2-pyridine carbonitrile (2.00 g, 19.2 mmol, 1 eq.), 5-amino-2-pyridine carbonitrile (1.14 g, 9.6 mmol, 0.5 eq.) and sulphur (0.60 g) were successively dissolved in ethanol (9 mL) during nitrogen gas purge. Hydrazine monohydrate (5 mL, 103 mmol, 5.4 eq.) was added dropwise via injector to the flask in an ice bath (0°C). The reaction mixture (black) was stirred at room temperature for 2 hours. Then the orange reaction mixture was heated to 90°C and refluxed for 2 h. The reaction mixture was then cooled to 0°C and the solid filtered through crucible and washed with ethanol (3 × 15 ml) and dried under vacuum. The matter was purified with column chromatography; chloroform / ethanol (15: 1). Characterization of matter was made by ^{1}H-NMR.

3.3.3 Photooxidation of PPA-dHTz

dHTz (2 mg, 7,896 mmol, 5 eq.) in 0.4 mL DMSO-d_6 and Methylene Blue (0.5 mg, 1.579 mmol, 1 eq.) dye molecule (photo-stimulant) in 0.4 mL DMSO were dissolved in NMR tube. The solution was illuminated at different temperatures in white light (LED, 100 W) in NMR tube at room temperature. Before illumination (t = 0 h) and then at t = 2 h and t = 8 h, the products were analyzed by NMR spectra (Figure 4.3).

3.3.4 Modification of PNIPAAm with amino dihydrotetrazine (PPA-dHTz)

PNIPAAm-COOH (300 mg, 0.163 mmol, 1 eq.) was transferred into a flask and dissolved with 4 mL of dichloromethane. NHS (0.157 g, 1.36 mmol, 10 eq.) was added after dissolved in 3 mL dichloromethane. EDC (0.260 g, 1.35 mmol, 10 eq.) was added and stirred along 30 min. Finally, dHTz (69 mg, 0.272 mmol, 2 eq.) was added and the reaction was continued overnight, after which time the reaction was terminated. The

matter was dialyzed in methanol and solvents were evaporated to dry by rotary evaporation.

3.3.5 Synthesis of *trans*-5-hydroxy-1-cyclooctene (TCO)

Trans-cyclooctenol is obtained from two isomers of polar (TCO-P) and apolar (TCO-A) as a result of the photoisomerization reaction of cis-cyclooctenol which is widely used in the literature [78]. *cis*-5-Hydroxycyclooctene (1 g, 7.92 mmol) and methyl benzoate (1.1 g, 8.06 mmol) were dissolved in 50 mL of diethyl ether/hexane mixture (9:1, v/v) in a Quartz tube. The tube was closed tightly and exposed to light at 254 nm in a cylindrical photoreactor equipped with lamps (16 × 8 W). After a 2-hour irradiation, the reaction mixture was passed through silica column containing silver nitrate (10% w/w) and eluent was collected into the Quartz tube again. Then the eluent containing remaining unmodified *cis*-5-hydroxycyclooctene was irradiated at 254 nm again and passed through the same column.

This irradiation-passing-through-column cycle was repeated 24 times. After 48 h of overall irradiation time, the product, TCO, was adsorbed on silver ions on the column, but *cis*-isomer was not. Finally, the column was washed with excess diethyl ether and the column content was emptied into 80 mL of ammonium hydroxide and the mixture was stirred for 30 min. The products containing two different isomers were extracted with diethyl ether (3 × 30 mL). The organic layers were dried over $MgSO_4$ and evaporated. The isomer mixture was separated by silica column (hexane/ethyl acetate, 4:1) yielding polar (255 mg) and apolar (226.5 mg) *trans*-isomers. Consequently, polar isomer was benefited more.

3.3.6 Synthesis of succinimidyl ester of *trans*-5-hydroxycyclooctene (TCO-NHS)

Briefly, polar TCO (100 mg, 0.792 mmol) and triethylamine (240 mg) were dissolved in 3 mL of acetonitrile at 0 °C. *N,N'*-disuccinimidyl carbonate (DSC) (608 mg, 2.376 mmol) was added under N_2 atmosphere to the solution. [76] The reaction was stirred at room temperature for 24 hours. After the reaction, water was added (30 mL) and the product was extracted with ethyl acetate (3×30 mL). Organic layers were combined and washed once with saturated sodium chloride solution (20 mL). The solvent was dried using $MgSO_4$ and evaporated on a rotary evaporator. The product was purified by silica column using ethyl acetate/hexane (2:1 v/v). Yield: 48%.

3.3.7 Preparation of glass surfaces

To bind protein or different polymer molecules to glass surfaces, firstly the surface must be purified from organic molecules. For this process, 50 pieces of glass were treated with potassium hydroxide solution (3 M) and cleaned with Piranha Solution respectively. Glasses were transferred in a flask, which was containing 50 mL of 3 M KOH solution and stirred gently through a magnetic stirrer. Glass surfaces were treated with KOH for 2 hours. Then, the potassium hydroxide solution was removed the flask. Glass surfaces were washed with distilled water and dried under the vacuum. Piranha Solution was added on glass surfaces and stirred for 1 hour. Then, the glasses were washed with enough distilled water five times and dried under vacuum (These glasses were labelled as **C0**). (**Attention:** The highly dangerous and explosive Piranha Solution was slowly poured in some ice taken into a clean beaker and neutralized with 1 M NaOH).

Glass surfaces were treated with 30 mL of ethanol solution, which was containing 16.7% APTES by volume along 24 hours at 60 ºC to form amine groups on the surface. The modified glass surfaces (**C1**) washed with ethanol (5 × 30 mL) and dried under vacuum. Previous works reported that APTES density was determined as averagely 2.1-4.2 amino groups per square nanometer [79]. Therefore, the mole amount of amino groups per glass surface (for both surfaces A = 6.28 cm^2) was calculated as 2.19-4.38×10^{-9} mol. For next modifications, this information was used. Amino groups were converted to carboxylic acid groups by succinic anhydrate to bind groups such as polymer or protein to amino functional glasses and to prepare functional surfaces [80]. For this purpose, 25 mL of dry dichloromethane containing succinic anhydrite (365 mg, 0.0036 mol) and triethylamine (200 µL, 0.0018 mol) was added to amino functional glass surfaces (23 pieces). Nitrogen gas was bubbled through the solution and the mixture was allowed to stir gently overnight. Glass surfaces (**C2**) were washed with dichloromethane (4×20 mL) and dried under vacuum.

3 M KOH preparation: KOH (8.42 g, 0.15 mol) was weighed into a 100 mL flask and dissolved by adding 50 mL of distilled water.

Preparation of Piranha Solution: Hydrogen peroxide and sulfuric acid were added with a 1:3 ratio at a well-dried flask respectively, with a total volume of 50 mL.

Preparation of 16.7% APTES solution: 5 mL of APTES (3-amino propyltriethoxysilane) was transferred to a flask and 25 mL of toluene was added and mixed well.

Conjugation of TCO to carboxylic acid functional glass surfaces

10 pieces of carboxylic acid functional glasses were placed in a flask and 8 mL of dry THF was added. DCC (4 mg, 1.93×10^{-5} mol) and DMAP (5 mg, 4.08×10^{-5} mol) were dissolved with THF and added to the flask containing glasses. Finally, TCO (10 mg, 7.92×10^{-5} mol) dissolved in 2 mL THF was added to the reaction flask. The reaction was allowed to stir at a slow speed with a magnetic stirrer and the reaction was continued for two days. At the end of the time, the reaction solvent was removed, the glasses were washed with THF (15 mL × 4) and dried under vacuum (**C3**).

dHTz binding on glass surfaces

Firstly, EDC (45 mg, 2.36×10^{-4} mol, 5 eq.) and NHS (27 mg, 2.36×10^{-4} mol, 5 eq.) were dissolved in 10 mL DMF, then triethylamine (TEA) (10 µL, 9.46×10^{-5} mol, 2 eq.) was added and mixed overnight. After, dHTz (12 mg, 4.73×10^{-5} mol, 1 eq.) was dissolved with DMF(10 mL) and added under N_2 atmosphere and mixed overnight. Finally, 6 pieces of glass surface with carboxylic acid functionalized (nearly $9{,}46\times10^{-9}$ mol functionalized group) were washed with DMF and CH_2Cl_2 three times (3× 15 mL for each solvent).

Grafting PNIPAAm-dHTz to glass surfaces via light-induced IEDDA

dHTz functional poly(N-isopropylacrylamide) (PNIPAAm-dHTz) (0,42 µmol, 5 eq.) and Methylene Blue (0,08 µmol, 1 eq.) with dHTz end groups were weighed in separate containers and dissolved in DMF and the required amount was taken. This prepared solution was added to the Petri dish to cover 5 pieces of TCO functional glass and the glass samples were irradiated with white LED light (100 W) for three hours. At the end of the period, the glasses were successively washed with methanol, THF and dichloromethane, dried under vacuum for one hour. The top surface was marked as the actual reaction that took place on the upper surface of the glass during the operations.

3.3.8 Preparation of protein-polymer conjugates

3.3.8.1 Synthesis of ConA- TCO-PNIPAAm conjugate

Concovalin A (50 mg, 0,000446 mmol, 1 eq.) was weighted into a flask and dissolved with 10 mL of PBS buffer solution, and pH was fixed at 9 with $NaHCO_3$. TCO-NHS(2 mg, 0,0044 mmol, 10 eq.) was weighted in a tube and dissolved in 200 μL DMF, then was added in protein solution. Reaction mixture was stirred in ice bath around 1 hour, and it was dialysed with distilled water along night. Half of the reaction mixture, which contain nearly 25 mg protein, was transferred into a flask, dHTz- PNIPAAm (1.1 mg, 0,000446 mol, 2 eq.) was added to the reaction. Methylene blue (0,14 mg, 0,000446 mol, 2 eq.) dissolved in 10 mL water and 1 mL was taken from this mixture and added in reaction. The solution was illuminated in white light (LED, 100 W) in flask at room temperature and analyzed with MALDI-TOF.

3.3.8.2 Synthesis of BSA-TCO-PNIPAAm conjugate

BSA (60 mg, 0,0009 mmol, 1 eq.) was weighted into a flask and dissolved with 5 mL of PBS buffer solution, and pH was fixed at 9 with $NaHCO_3$. TCO-NHS(4 mg, 0,009 mmol, 10 eq.) was weighted in a tube and dissolved in 200 μL DMF, then was added in protein solution. Rxn mixture was stirred in an ice bath around 2 hours, and it was dialysed with distilled water along the night. Half of the reaction mixture, which contain nearly 30 mg protein, was transferred into a flask, dHTz- PNIPAAm (2.2 mg, 0,000446 mol, 2 eq.) was added to the reaction. Methylene blue (0,28 mg, 0,000446 mol, 2 eq.) dissolved in 10 mL water and 1 mL was taken from this mixture and added in rxn. The solution was illuminated in white light (LED, 100 W) in flask at room temperature and analyzed with MALDI-TOF.

4. RESULTS AND DISCUSSION

4.1 Photooxidation of dHTz

PPA-dHTz was synthesized successfully according to the NMR spectrum (Figure 4.1). Then it was employed in photooxidation in order to understand its turn on capability in light-induced IEDDA reaction (Figure 4.2).

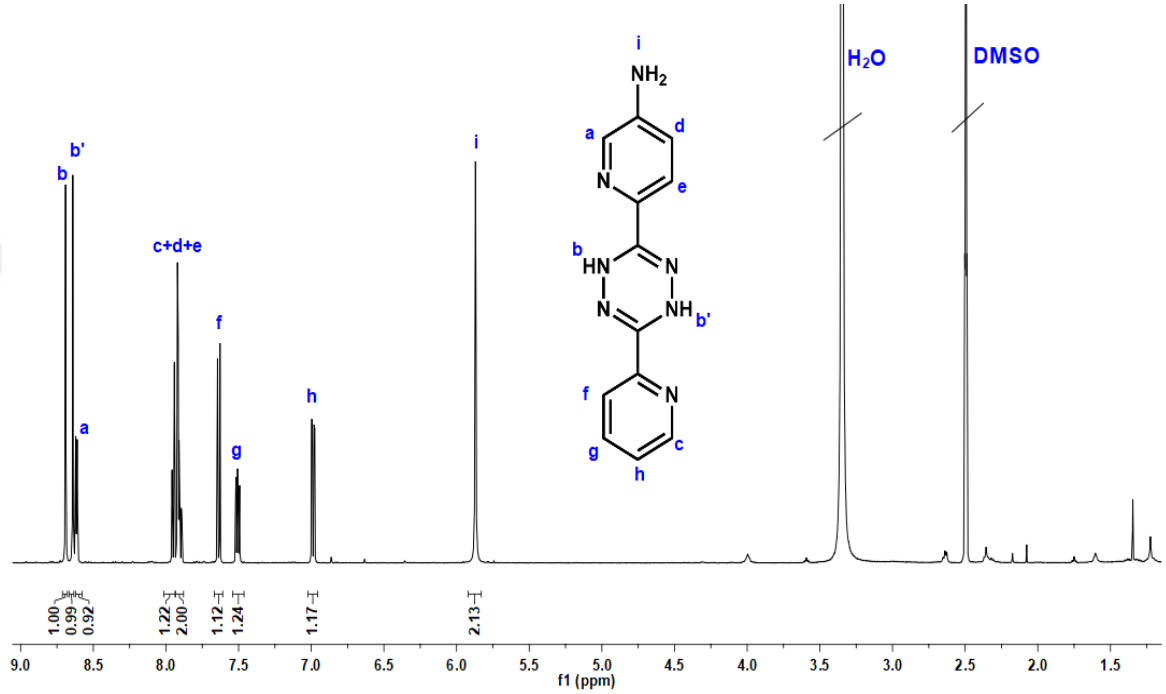


Figure 4.1 : ^{1}H-NMR spectrum of 6-(6-(pyridin-2-yl) -1,4-dihydro-1,2,4,5-tetrazine-3-yl) pyridin-3-amine (PPA-dHTz) (DMSO-d_6).

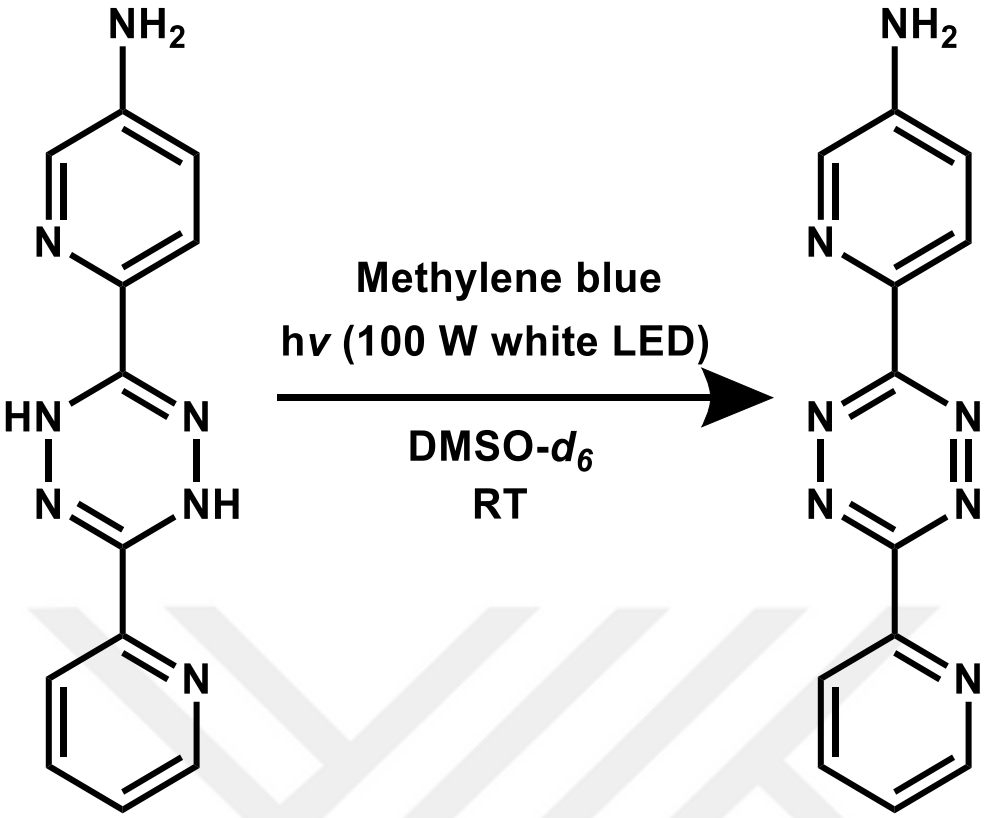


Figure 4.2 : Photooxidation of dHTz.

The NMR spectra in Figure 4.3 contain the dHTz molecule and the tetrazine molecule formed by illuminating the white light (LED, 100W) for 2 and 8 hours in the presence of Methylene Blue [81]. After two hours of illumination, peaks belonging to the tetrazine molecule appeared and at the end of eight hours the intensity of the peaks increased. It was also observed that the secondary amine proton peaks in the dHTz molecule marked with b decreased and the peaks belonging to the primary amine group shifted to a low area. These results show that the tetrazine molecule can be successfully formed in situ with light.

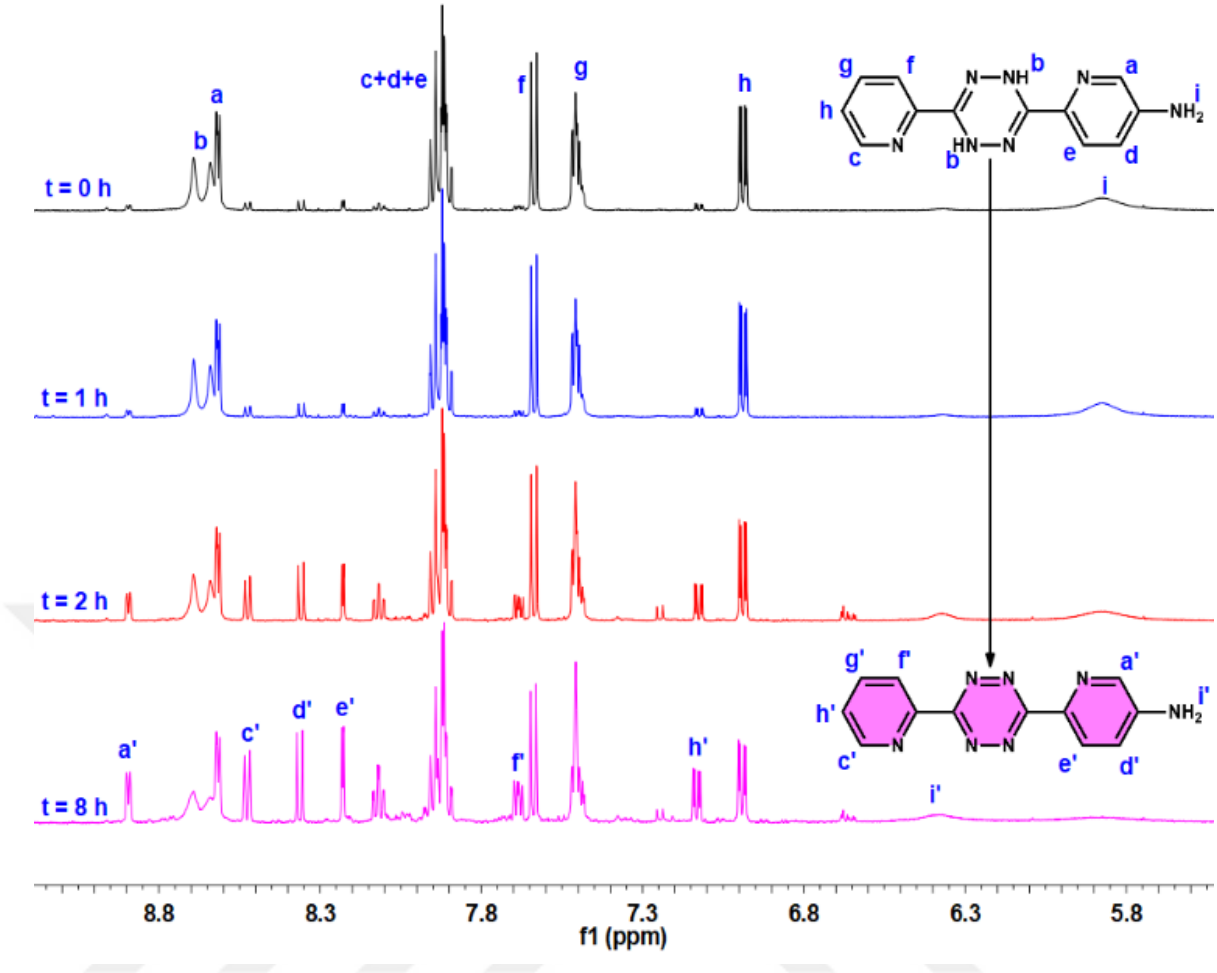


Figure 4.3 : ^{1}H-NMR spectra of a DMSO-d_6 solution containing PPA-dHTz and Methylene Blue at various time of irradiation with white LED (100 W).

4.2 Synthesis of PNIPAAm-COOH

The PNIPAAm-COOH had a gravimetric monomer conversion rate of 64.5% and a theoretical molecular mass of 2190 g/mol according to its ^{1}H-NMR spectrum of as shown in Figure 4.4. For molecular mass calculation from NMR end group analysis, ratio of peak integrations of PNIPAAm (4.00 ppm, peak b in Figure 4.4) and the CTA-COOH (3.36 ppm, peak c in Figure 4.4) was used. The molecular mass was determined with GPC (DMF as eluent) and NMR analysis as $M_{n,GPC}$ = 3420 g/mol (M_w/M_n = 1.07) and $M_{n,NMR}$ = 2510 g/mol respectively.

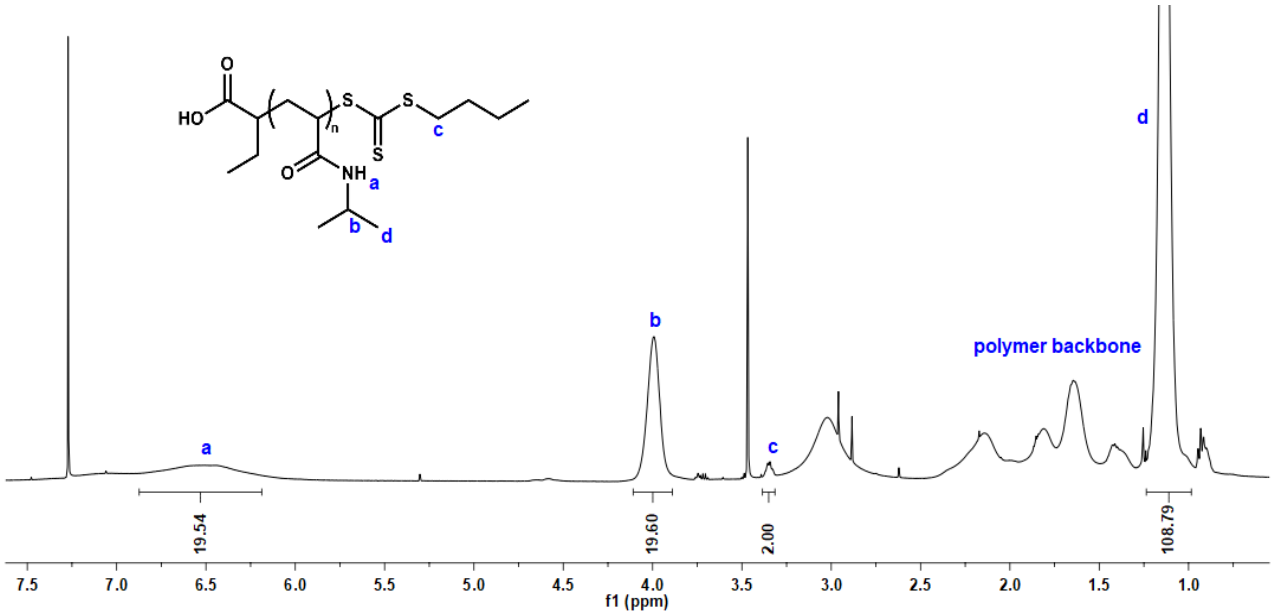


Figure 4.4 : ^{1}H-NMR spectra of PNIPAAm-COOH ($CDCl_3$).

4.3 Modification of PNIPAAm with Amino Dihydrotetrazine (dHTz-NH_2)

Successful synthesis of the product (Figure 4.5) is understood from the ^{1}H-NMR spectrum containing all characteristic peaks of the product. In the GPC (eluent DMF) analyzes, molecular weights were determined as $M_{n,GPC}$=4400 g/mol; M_w/M_n = 1.07; $Mn_{,NMR}$ = 2830 g/mol) The ^{1}H-NMR spectrum of matter is given in Figure 4.6. This product was used in glass surface modifications.

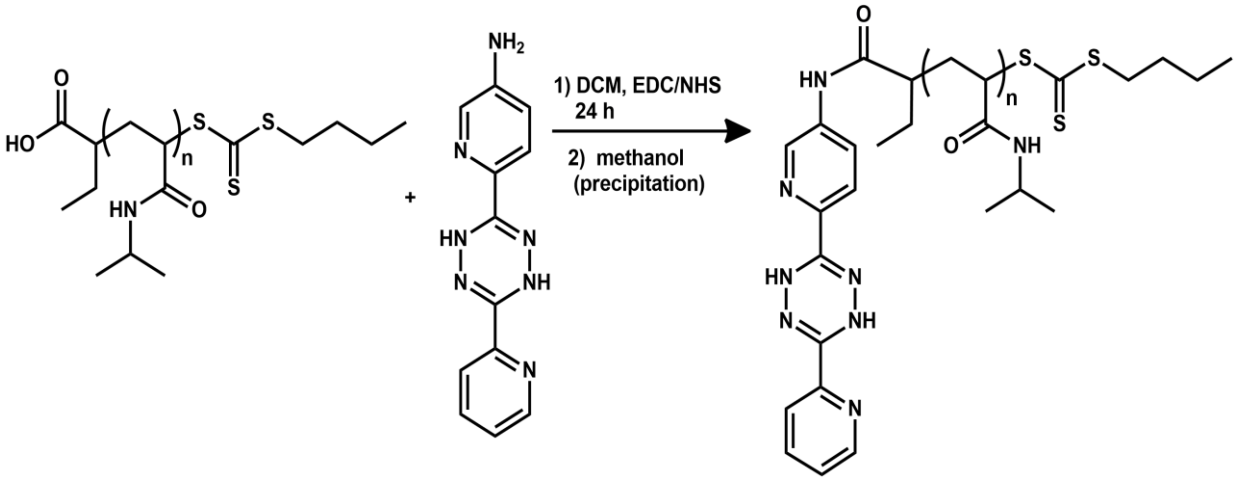


Figure 4.5 : Modification of PNIPAAm with PPA-dHTz.

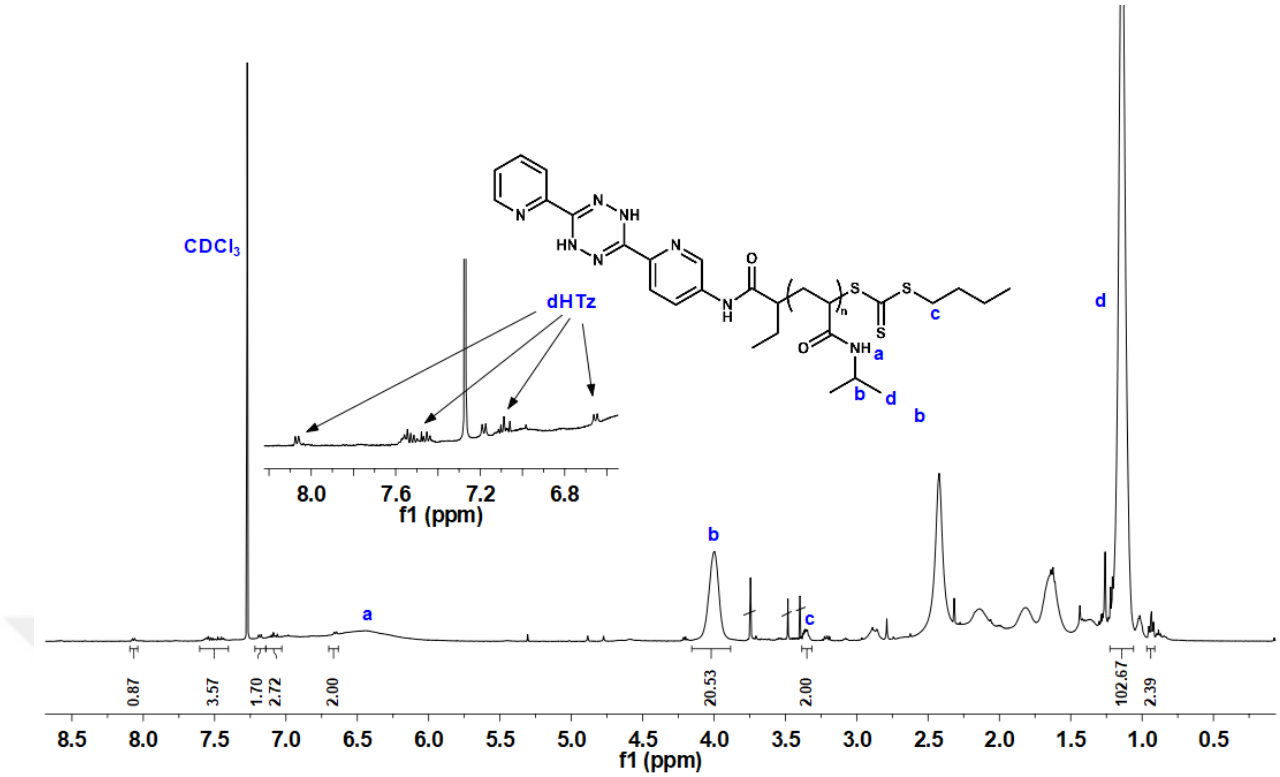


Figure 4.6: ^{1}H NMR spectra of PNIPAAm-dHTz ($CDCl_3$).

4.4 Characterization of TCO Derivatives

TCO and TCO-NHS were synthesized successfully and characterized through ^{1}H-NMR. The ^{1}H NMR spectrum of TCO shown in Figure 4.7, proved the typical NMR peaks linked to methylene protons of octene ring, (m, 10H, -CH_2-) from 1.45 to 2.36 ppm). Hydroxyl group proton appears at 3.41 ppm (m, 1H, HO-CH-), also characteristic dien signals are visible at 5.39 (m, 1H, CH=CH-) and 5.58 ppm (m, 1H, -CH=CH-), which are all related to specific peaks of the desired molecule.

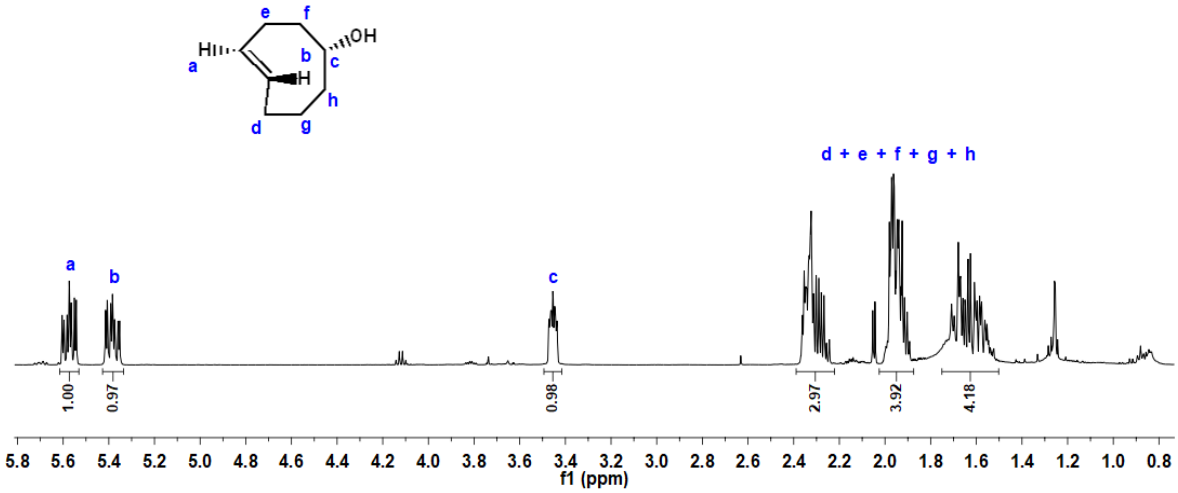


Figure 4.7 : ^{1}H-NMR spectra of TCO ($CDCl_3$).

In addition to all these characteristic peaks of TCO, methylene signals belonging to the NHS functional group, which can be seen at 2.84 ppm (-CH_2-CH_2-), show that TCO-NHS has been successfully synthesized (Figure 4.8).

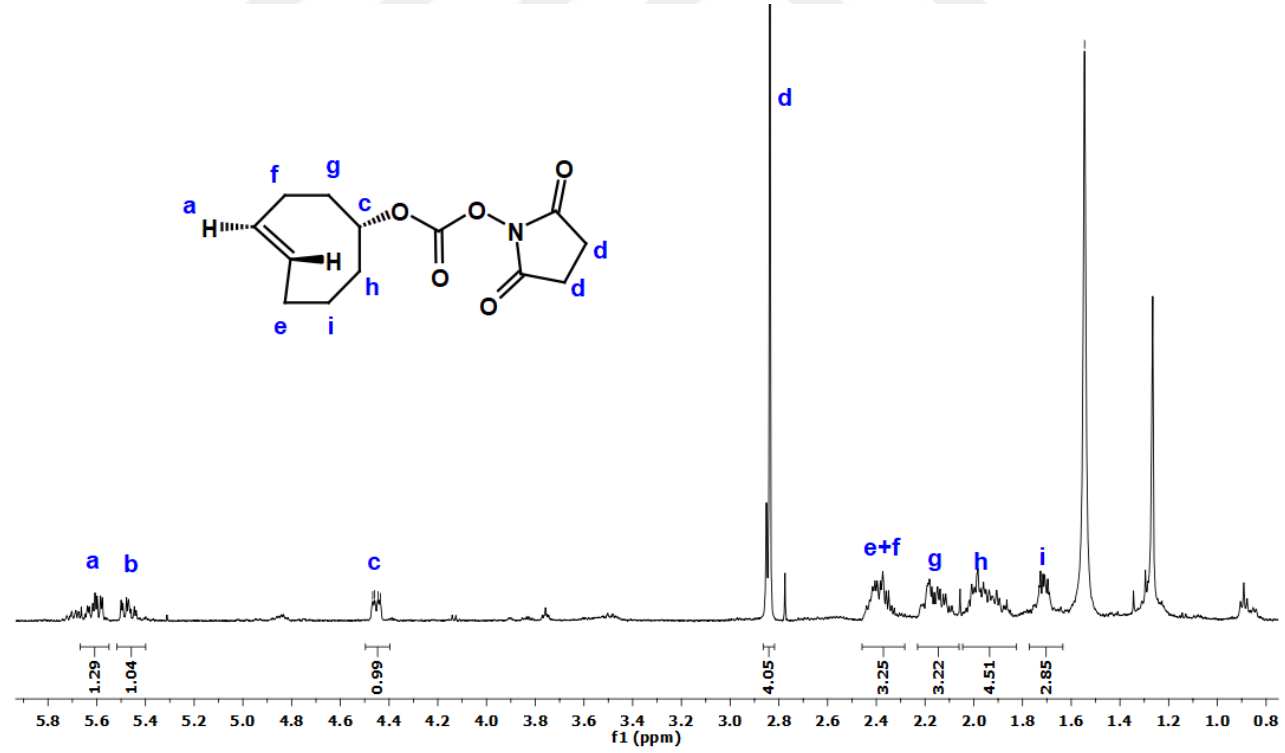
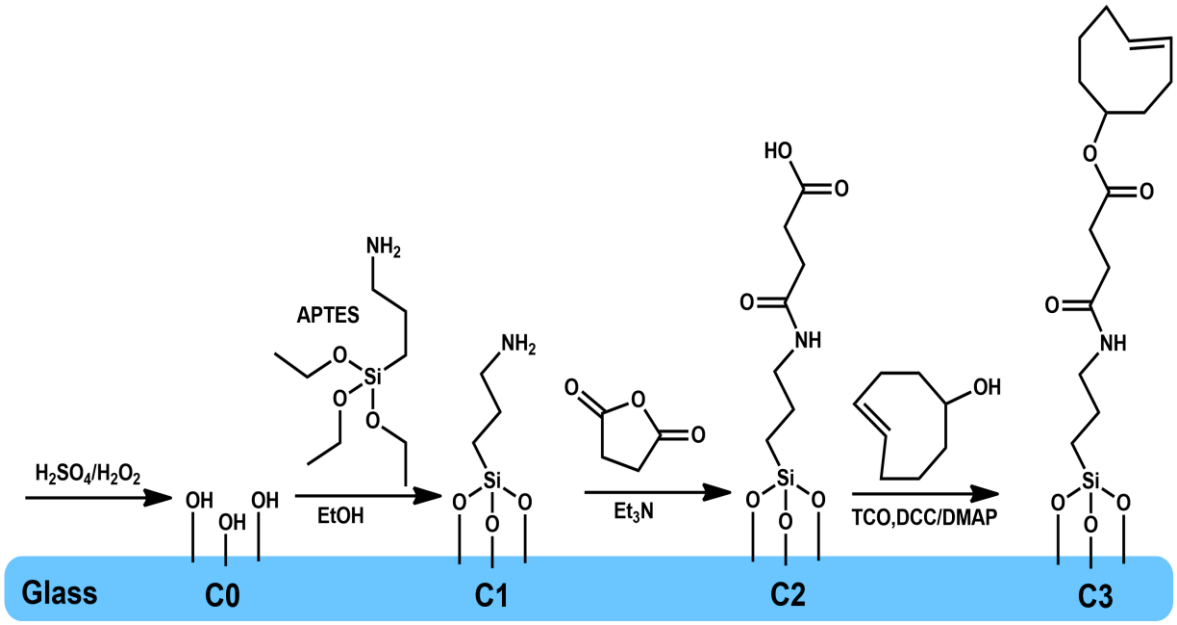


Figure 4.8 : ^{1}H-NMR spectra of TCO-NHS ($CDCl_3$).

4.5 Modification of Glass Surfaces

Two distinct glass surfaces were prepared for two distinct grafting strategy. In the first strategy, glass surfaces were modified with TCO for grafting polymer. While, the second strategy involves modification of glass surfaces with PPA-dHTz for grafting protein containing TCO group (i.e. Concanavalin A-TCO). A straightforward modification route was followed as shown in Figure 4.9 (upper part). Circular glass samples (*d*: 1 cm) were cleaned with Piranha solution and KOH solution successively (C0), and treated with APTES to obtain amino functionalities on the surfaces (C1). Then, glass samples were reacted with succinic anhydride in the presence of trimethylamine to obtain glass surfaces exposing carboxylic acids (C2).

Next, carboxylic acids were utilized in Steglich Esterification of TCOs (C3). TCO-modified glass surfaces were, then, used in grafting PNIPAAm-dHTz and PLA-dHTz to the surfaces by photo-IEDDA reaction. In the second strategy, TCO and PPA-dHTz groups were exchanged. Firstly, carboxylic acid functional glass surfaces were decorated with PPA-dHTz through a classical carbodiimide mediated amidation in the presence of EDC/NHS, and subsequently, Concanavalin A-TCO conjugate was grafted to the surfaces by photo-IEDDA (Figure 4.9 lower part and Figure 4.11).



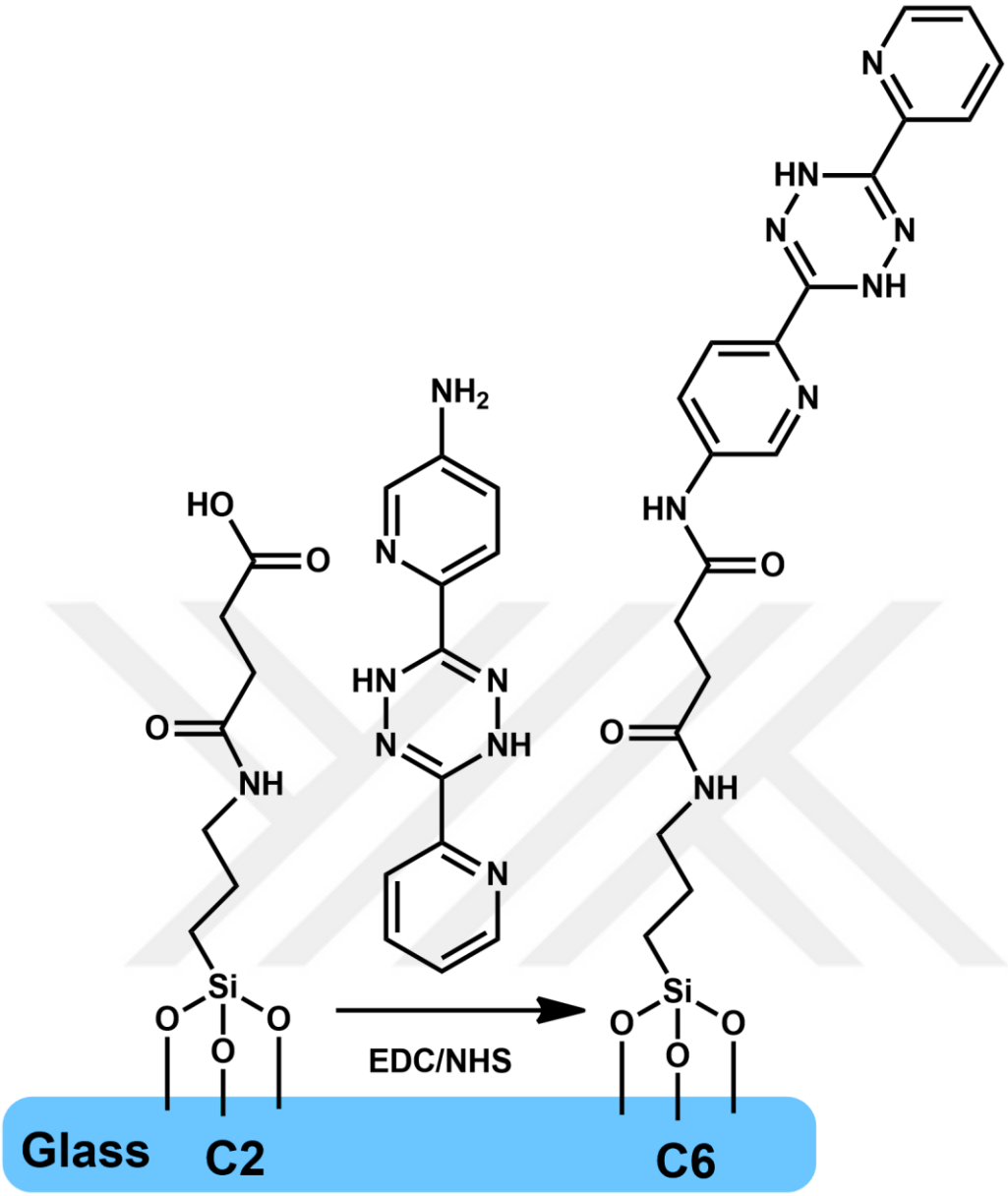


Figure 4.9 : Glass surface modification with Piranha(C0), APTES (C1), Succinic anhydrate(C2), TCO(C3), dHTz(C6) respectively.

For PNIPAAm-dHTz grafting, glass surfaces were covered with a DMF solution containing the polymer (49.2 µM) and photosensitizer, Methylene Blue (10.8 µM), and irradiated with white LED (100 W) for three hours (Figure 4.10).

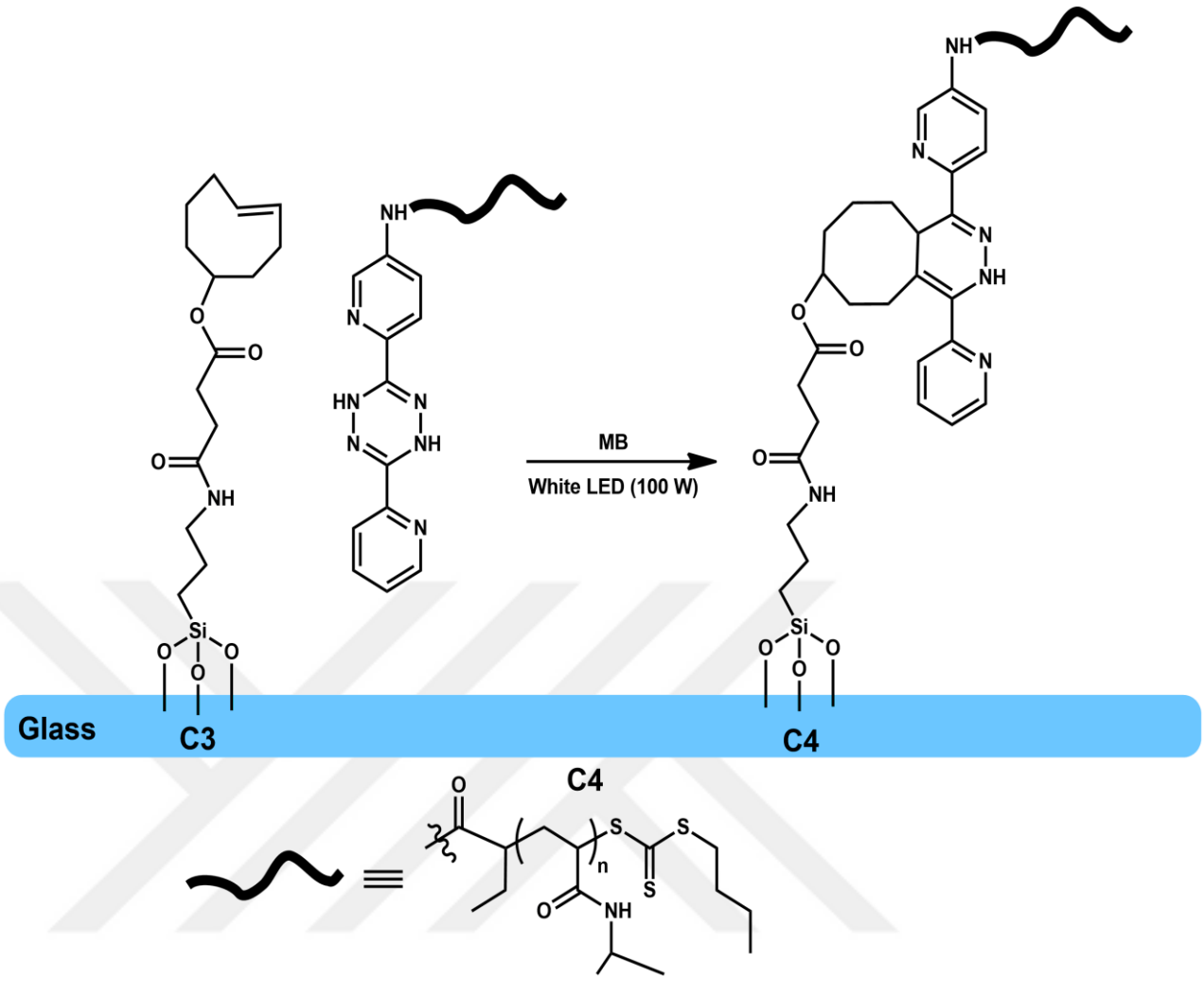


Figure 4.10 : Grafting PNIPAAm-dHTz to the glass surfaces.

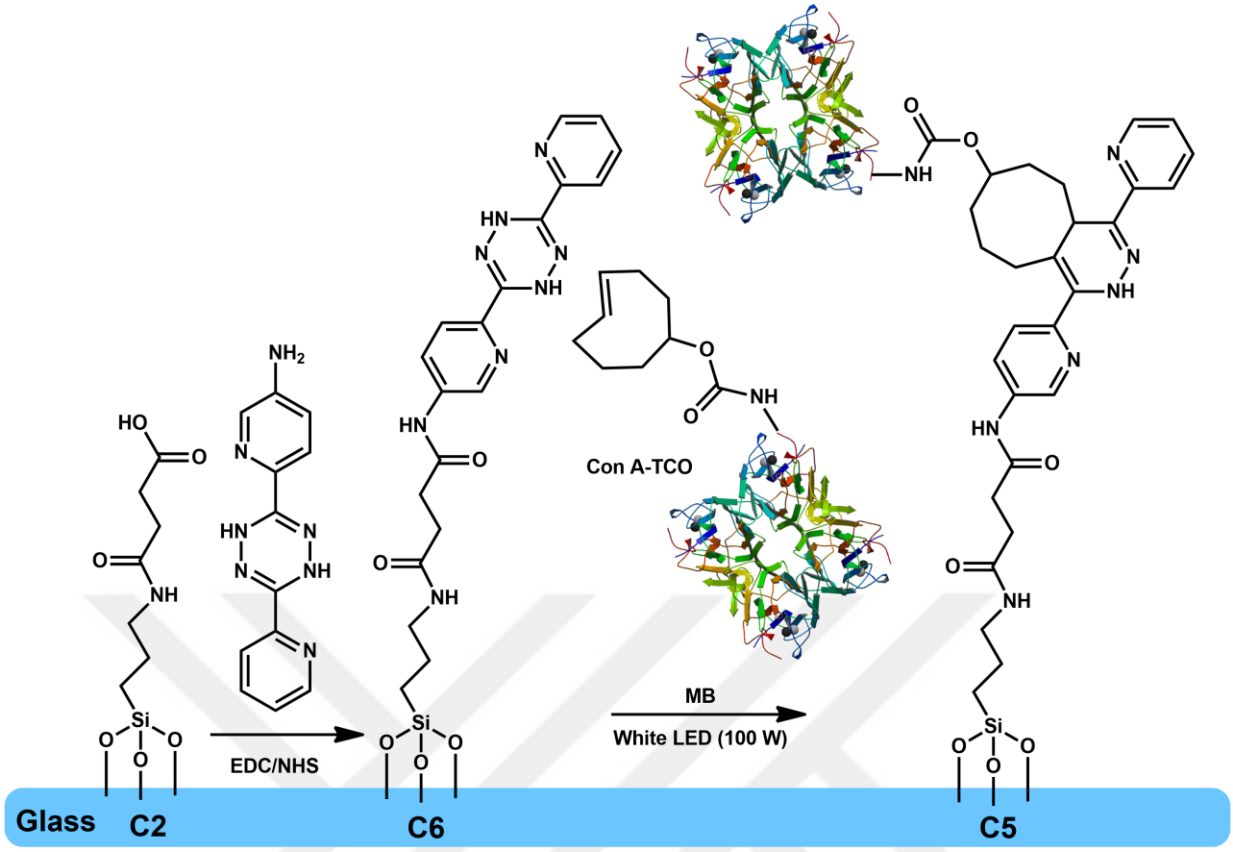


Figure 4.11: Grafting Con A onto glass surfaces.

4.6 Characterizations of Glass Surfaces

4.6.1 FT-IR analysis

As shown in Figure 4.12**,** FT-IR spectrum of unmodified glass surfaces **(C0)** did not contain significant bands after treatment with Piranha solution; thus, most of the organic impurities on the glass surface were removed. Glass samples treated by APTES **(C1)** exhibited characteristic C-H band at 2926 cm^{-1}, and a characteristic amine band centered at 3346 cm^{-1}. After modification with TCO, glass surfaces **(C3)** exhibited a typical carbonyl band at 1738 cm^{-1}. Grafting with PNIPAAm resulted in appearance of a broad band centered at 3346 cm^{-1} that could be assigned to N-H of PNIPAAm and O-H of residual water **(C4).** Moreover, typical isopropyl bands at 1377 and 1365 cm-1 and the methylene peaks at 2926 cm-1 appeared. In addition, the carbonyl band intensity increased and widened. In the FT-IR spectra of dHTZ modified glass surfaces **(C6),** C=N stretching band was observed around 1650 cm^{-1}, while Concanavalin A **(C5)** grafted surfaces exhibited ester and amide bands at 1740 cm^{-1} and 1644 cm^{-1}, and C-H bands at 2926 cm^{-1}.

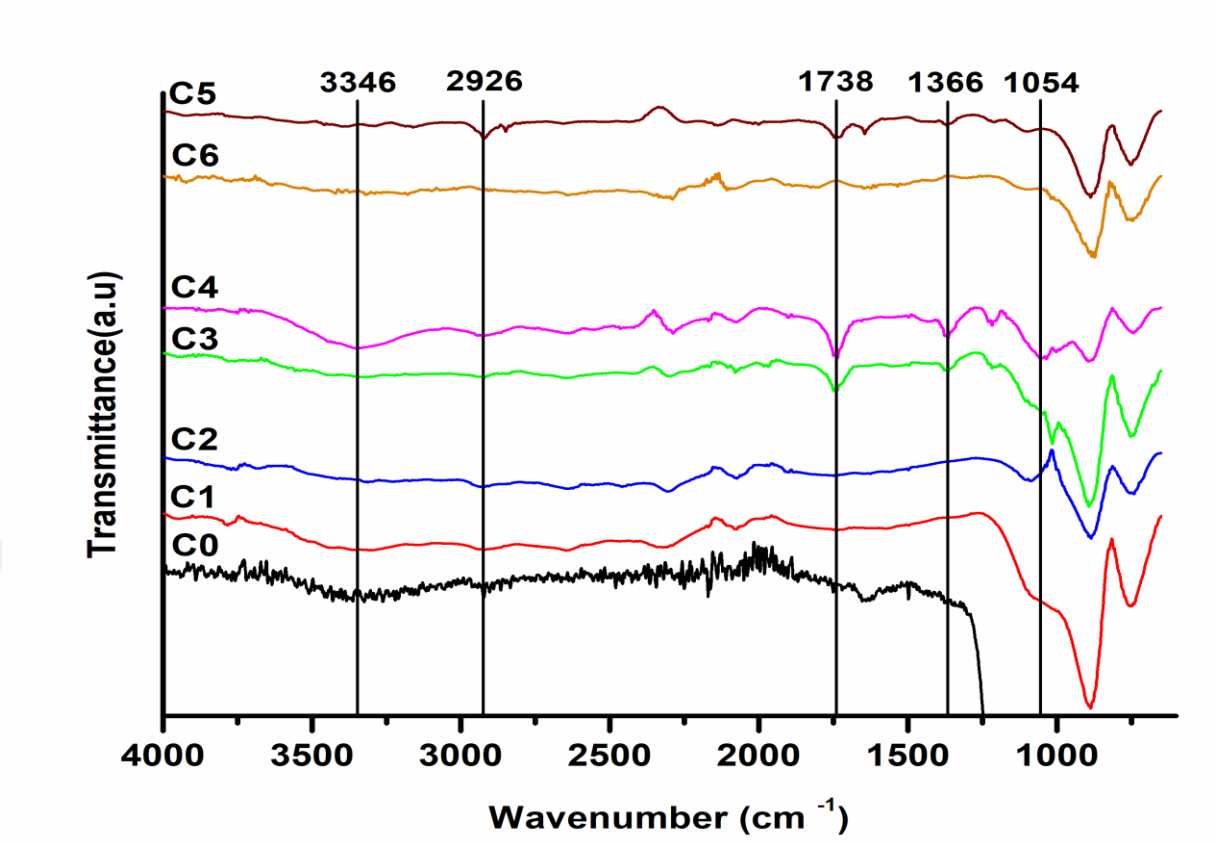


Figure 4.12 : FT-IR spectra of functionalized glass surfaces.

As mentioned in Figure 4.12**, C0**: after cleaning with Piranha and KOH solutions; **C1**: after silanization with APTES; **C2**: after reaction with succinic anhydride; **C3**: after functionalization with TCO; **C4**: after grafting PNIPAAm to the TCO-modified glass surface via photo-IEDDA; **C5**: after grafting Concanavalin A (Con A) to the dHTz-modified glass surface via photo-IEDDA; **C6**: after functionalization with dHTz.

4.6.2 Water contact angle measurements (WCA)

The results were supported by water contact angle measurements as displayed in Figure 4.13 and 4.14 The water contact angle of glass samples modified with APTES dramatically increased from 52º (**a-C0**; the glass surface cleaned with Piranha solution) to 96º **(b-C1)** because of hydrophobic nature of APTES [82]. After modification with succinic anhydride, the contact angle decreased to 66º **(c-C2)** due to formation of carboxylic acid functionalities.

Conjugation of hydrophobic TCO onto the surfaces increased the contact angle to 77º **(d-C3).** After grafting TCO modified glasses with PNIPAAm by photo-IEDDA reaction, the contact angle of the surfaces were determined as 71º **(e-C4).** Principally, properties of polymer-coated surfaces may vary depending on several factors such as grafting method, molecular mass and grafting density. In fact, the results obtained in the current study were consistent with the literature [83, 84]

In the case of dHTz modification of glass samples, contact angle was observed as 61º which was consistent with expected value since dHTz moiety with pyridyl substituents has higher hydrogen bonding capacity compared to the precursor, carboxylic acid group. When Concanavalin A was grafted to the dHTz modified surfaces via photo-IEDDA reaction, the contact angle decreased to 56º **(f-C5)** due to hydrophilic nature of the protein. According to earlier reports, coating relatively hydrophobic surfaces with proteins usually decreases contact angle [85].

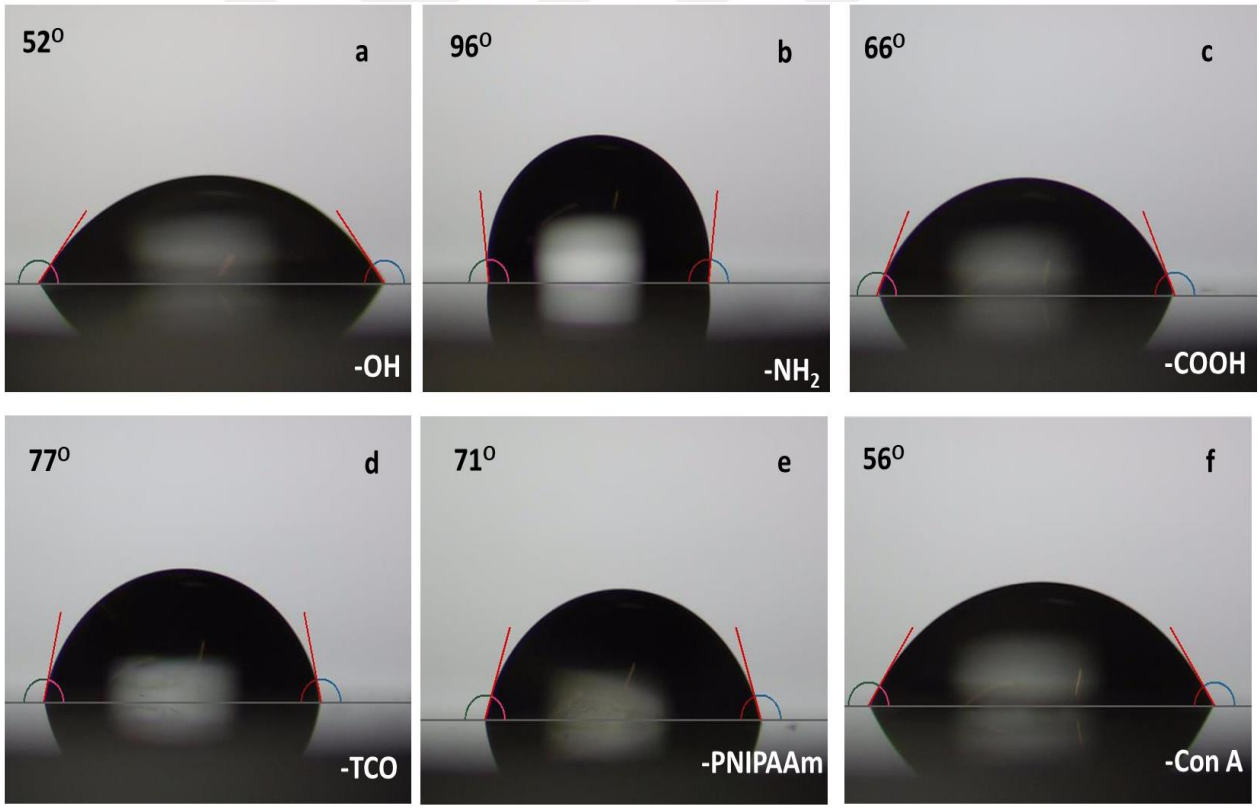


Figure 4.13: WCA of functionalized glass surfaces.

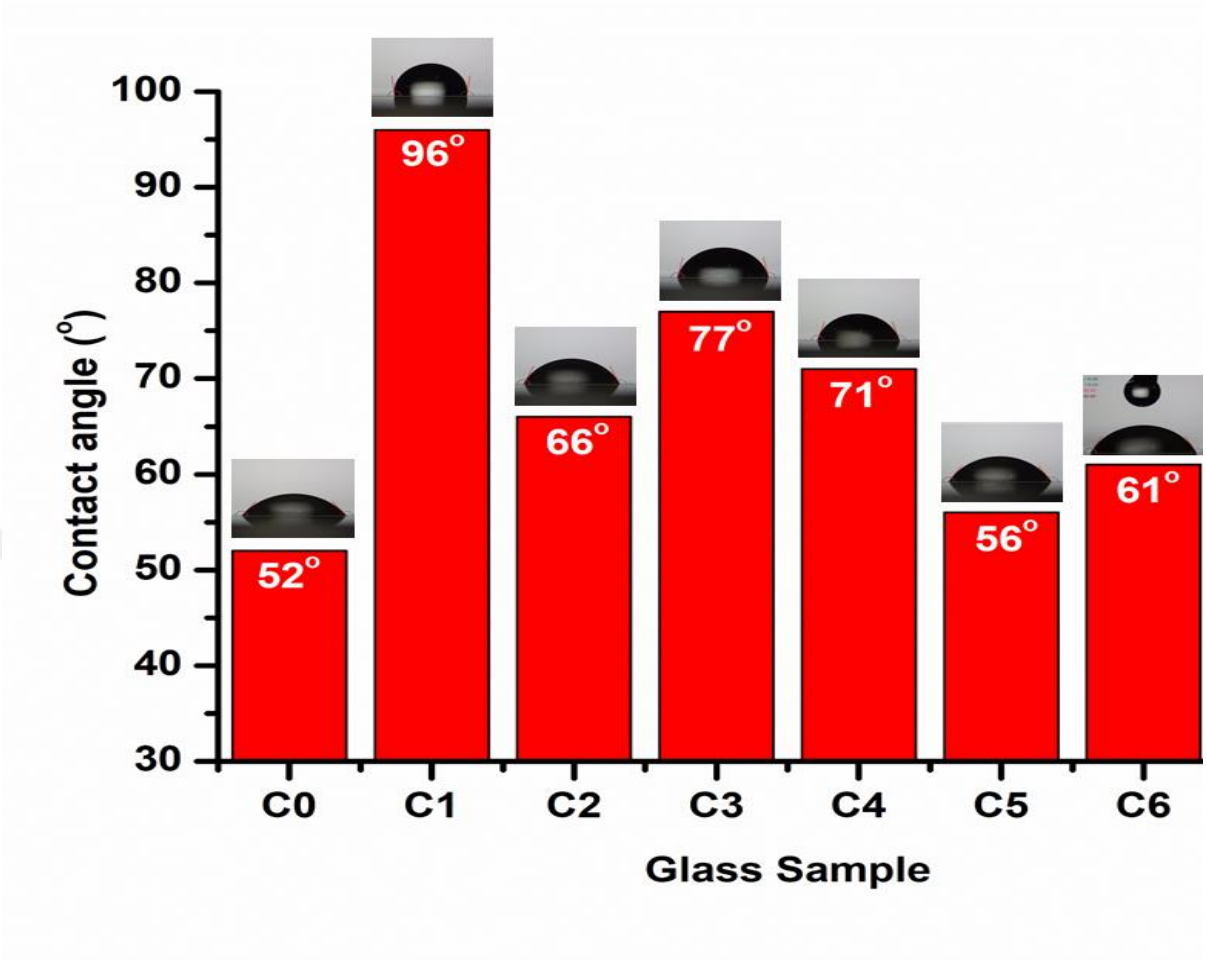


Figure 4.14 : Comparison of WCA of functionalized glass surfaces.

4.6.3 SEM analysis

The surface grafting was further evaluated by analyzing surface morphology with SEM as shown in Figure 4.15. The surface of glasses before grafting (**C2**) was smooth and had no impurity. After photo-IEDDA reaction between TCO-modified glass surface and dHTz functional polymer, roughness distributed whole over the surface was observed (**C4**). This roughness was attributed to grafting a layer of polymer which was more obviously observed on an accidentally scratched surface indicated with arrows in the SEM image of the PNIPAAm grafted (**C4**) surfaces.

Regarding the second strategy, the TCO and dHTz moieties were exchanged, and grafting a protein to glass surface was aimed. For this, dHTz-modified glass samples were covered with a solution of Concanavalin A-TCO and Methylene Blue and

exposed to LED light irradiation. After irradiation, aggregates of the Concanavalin A distributed on the surface were observed as shown in Figure 4.10 (**C5**). Similar morphology of surfaces grafted with a protein (i.e. BSA) was reported previously[86]. This indicated that glass surfaces were grafted with Concanavalin A successfully.

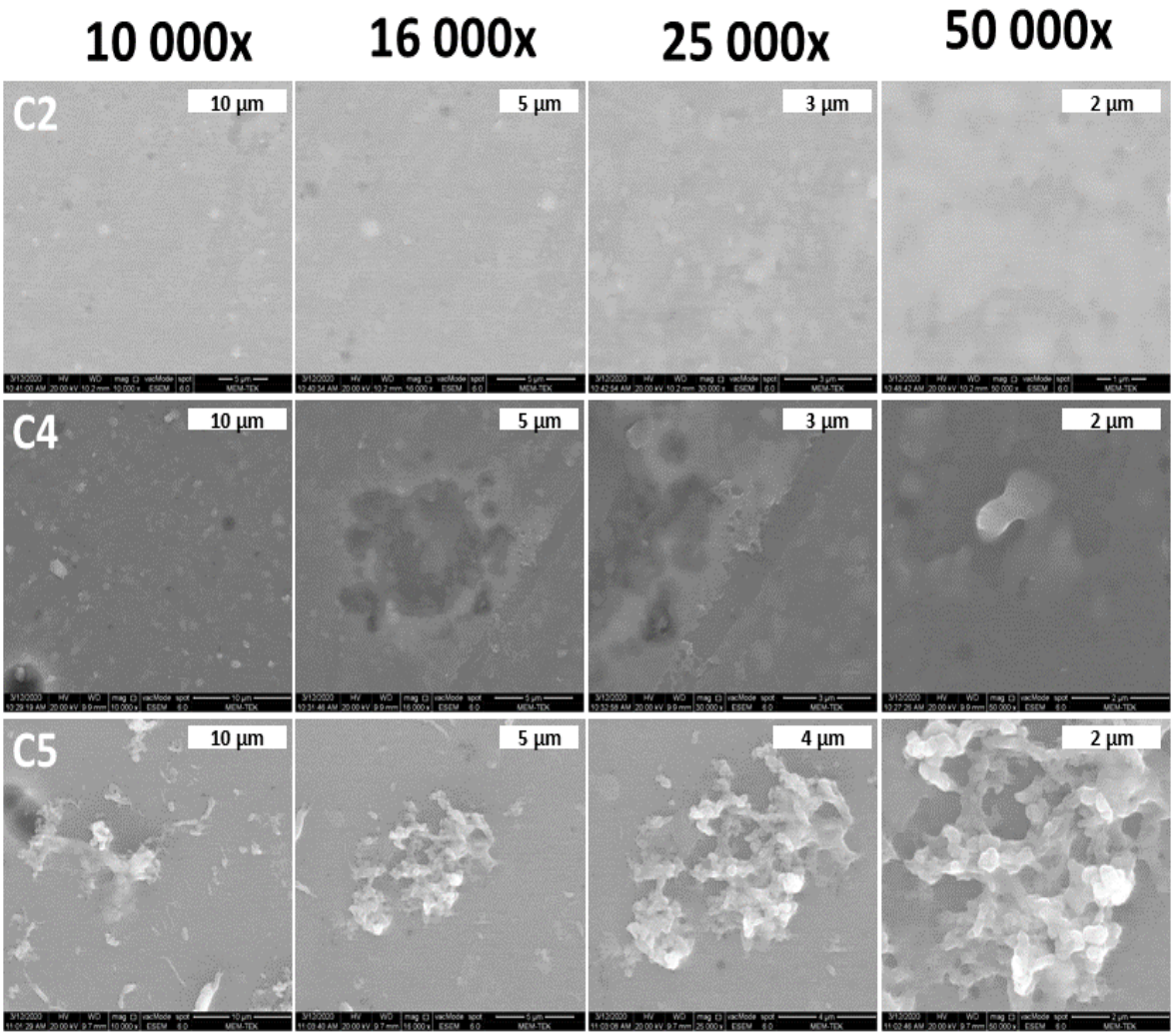


Figure 4.15 : SEM images of functionalized glass surfaces.

4.7 Preparation of Protein-Polymer Conjugates

The light induced IEDDa reaction was also employed in formation of protein-polymer conjugates. For this, Con A and BSA were selected as model proteins. As shown in Figure 4.16, a PBS solution containing PNIPAAm-dHTz, Con A-TCO and Methylene Blue was iradiated with white LED for photo-IEDDA yielding the conjugate.

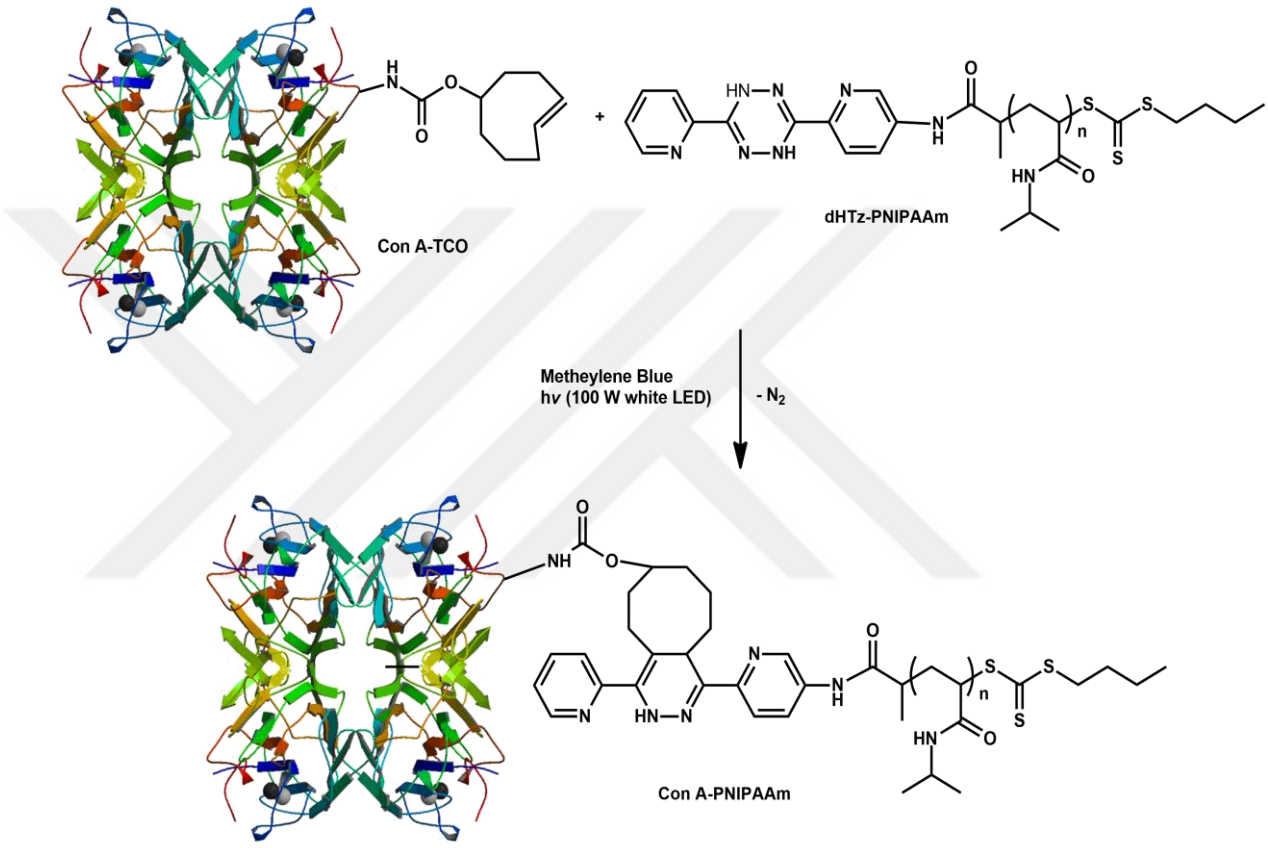


Figure 4.16: Schematic representation of Con A-PNIPAAm reaction.

In MALDI-TOF analysis, the band corresponding to protein was shifted to higher molecular masses and widened, after modification with TCO and photo-IEDDA.

The increase in the molecular mass peak is clearly seen after protein conjugation of the polymer (PNIPAAm-dHTz). This result is evidence for formation of the targeted protein-polymer conjugate (Figure 4.17).

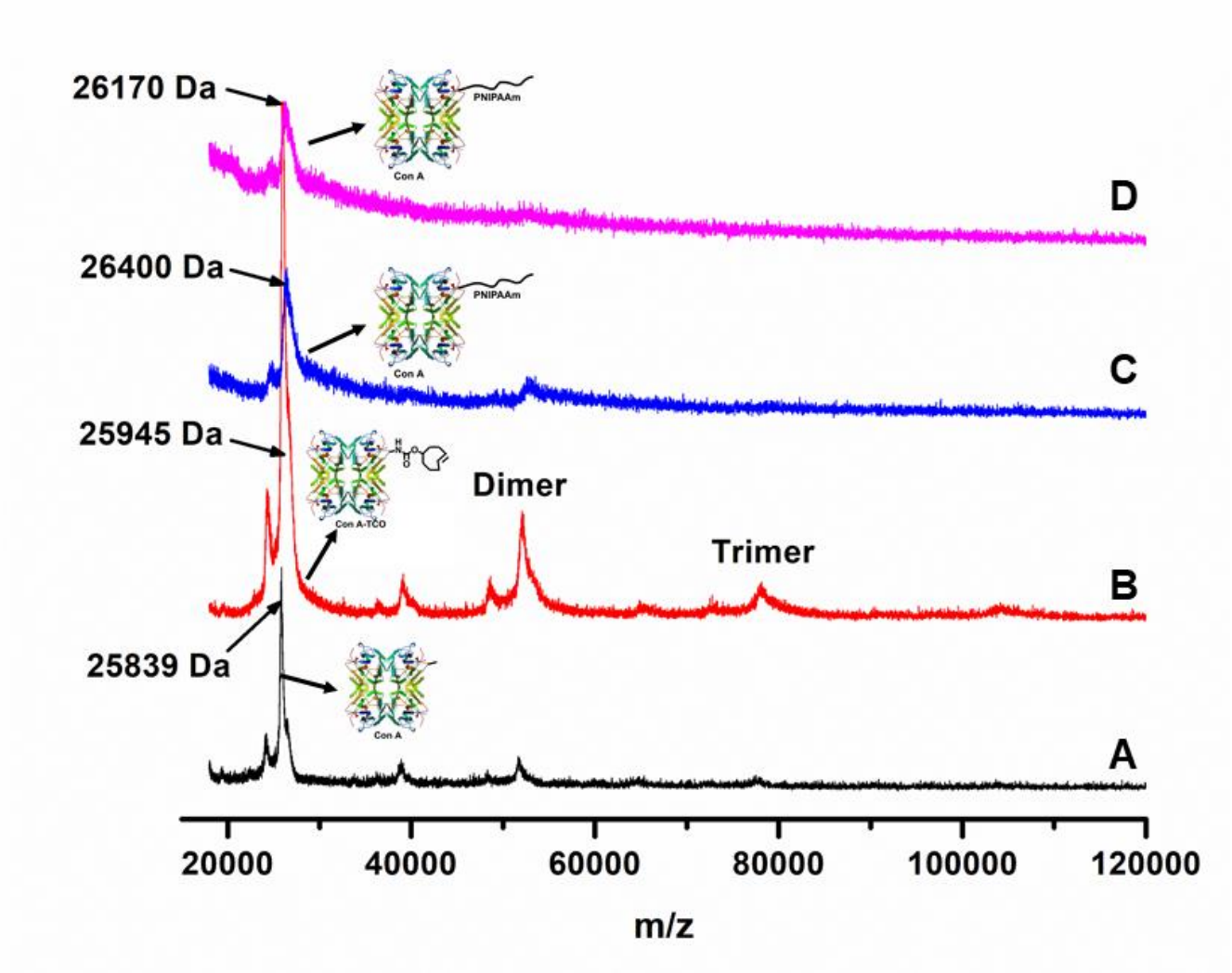


Figure 4.17 : MALDI-TOF mass spectra of (A) native Con A; (B) Con A modified with TCO; (C) Con A-PNIPAAm conjugate obtained via photo-IEDDA after 5 hours irradiation; (D) Con A-PNIPAAm conjugate obtained via photo-IEDDA after 24 hours irradiation.

Similar, mass spectroscopic behaviour was observed in bioconjugation reaction relying on light-induced IEDDA (Figure 4.18). The molecular mass was increased after photo-conjugation reactions. Both results supports successful conjugation of polymers to proteins.

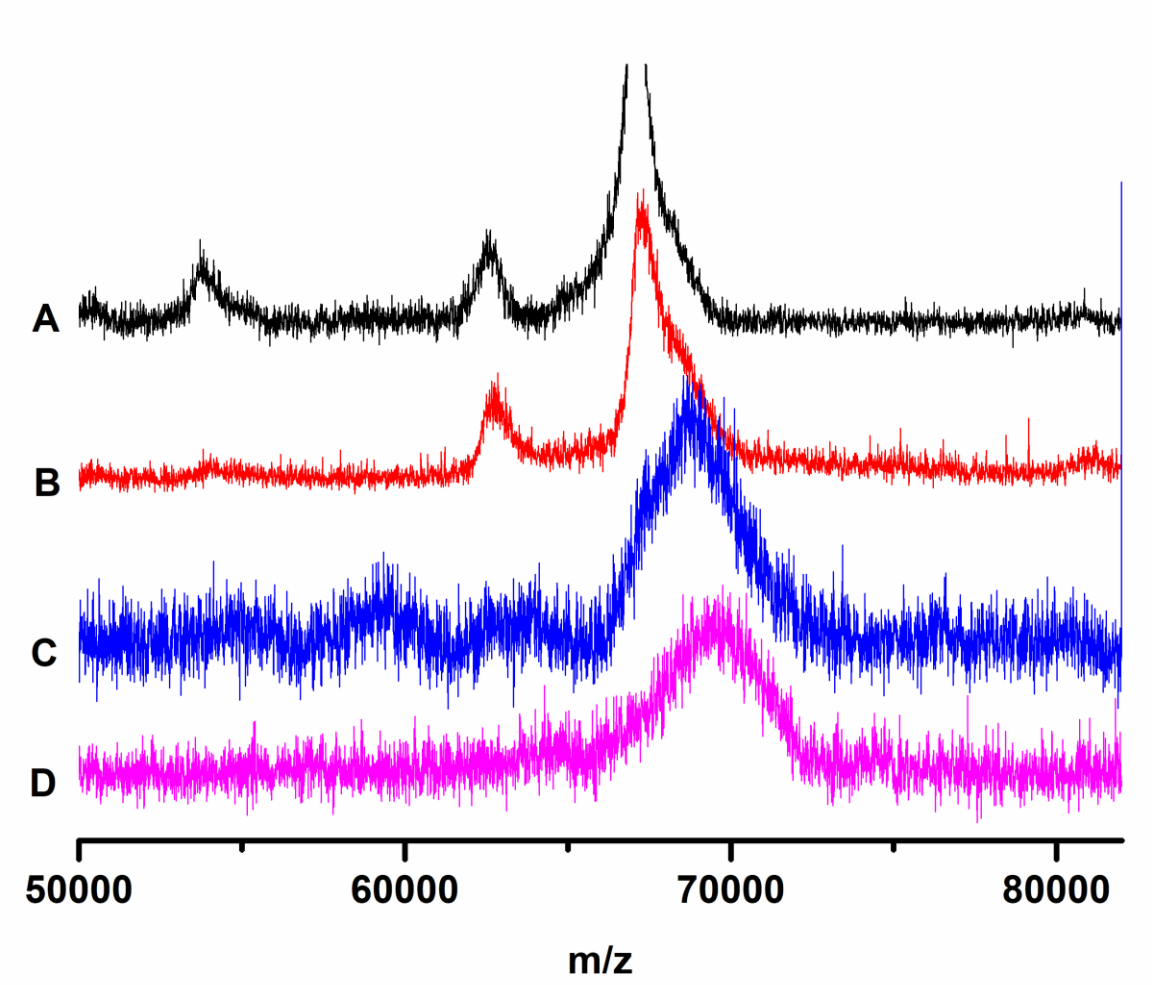


Figure 4.18 : MALDI-TOF mass spectra of (A) native BSA (4 mg/mL) with) α-cyano-4-hydroxycinnamic acid (**CHCA**) as a MALDI-TOF matrice; (B) native BSA (4 mg/mL) with 3,5-dimethoxy-4-hydroxycinnamic acid; (C) BSA-TCO; (D) BSA-PNIPAAm conjugate obtained via photo-IEDDA after 10 hours irradiation.

5. CONCLUSION

As a conclusion, a novel surface grafting approach based on light-induced inverse electron demand Diels Alder (photo-IEDDA) reaction was developed. The approach involves *in situ* photochemical formation of tetrazine molecule from a precursor, namely dHTz, and a subsequent IEDDA reaction of tetrazine moieties with *trans*-cyclooctene. The modification of glass surfaces for the first time with this method is the most important success criterion of this study. For grafting glass surfaces, two different methodologies were followed. In the first methodology, *trans*-5-hydroxycyclooctene (TCO) group containing glass surfaces were prepared for grafting polymers. Glass surfaces were cleaned to expose most of the terminal hydroxyl groups for use in chemically attaching TCO groups for grafting the polymers to the surfaces. dHTz end-functionalized PNIPAAm-dHTz was prepared by RAFT polymerization and post-polymerization with PPA-dHTz. Then, TCO-modified glass surfaces covered with a solution containing the dHTz functional polymers and Methylene Blue were exposed to visible light irradiation to achieve the photo-IEDDA reaction leading to grafting the polymers to the surfaces. In the second methodology**,** the light-induced approach was extended for grafting proteins to glass surfaces. Firstly, dHTz-modified glass surfaces were utilized instead of TCO-modified ones; thus, click handles on the surface and on the grafted molecule (protein) were exchanged. Similarly, another photo-IEDDA reaction was achieved on the dHTz-modified glass surfaces covered with a solution containing Concanavalin A-TCO and Methylene Blue by irradiation with white LED. Both polymer and protein grafted surfaces was successfully characterized by contact angle measurements, FT-IR spectroscopy and SEM analysis. Regardless of that if the surfaces were modified with TCO or dHTz, grafting macromolecules to surfaces achieved. The results indicated that the photo-IEDDA reaction can be employed in modification of surfaces as a novel light-induced grafting strategy. This recently developed strategy would offer **spatial** and **temporal** control in functionalization surfaces.

In addition to surface grafting, preparation of protein (e.g. Con A or BSA)-polymer conjugates with this method has been shown with the assitance of MALDI-TOF analysis. When the results are evaluated, we have demonstrated that the conjugation of protein and polymer can be achieved successfully with the proposed approach which

has great potential for preparation of protein-polymer conjugates by providing temporal and spatial control.

REFERENCES

[1] **Kolb, H. C., M. G. Finn , and K. B. Sharpless.** (2001). Click Chemistry: Diverse Chemical Function from a Few Good Reactions, *Angewandte Chemie International Edition*, ***40***(11), 2004-2021.

[2] **Lutz, J.-F. and H. G. Börner.** (2008). Modern trends in polymer bioconjugates design, *Progress in Polymer Science*, ***33***(1), 1-39.

[3] **DeForest, C. A., B. D. Polizzotti , and K. S. Anseth.** (2009). Sequential click reactions for synthesizing and patterning three-dimensional cell microenvironments, *Nature Materials*, ***8***(8), 659-664.

[4] **Selvanathan, A., V. O. Sara, M. Ngalle Eric, M. Christopher, et al.** (2013). Photo-click chemistry strategies for spatiotemporal control of metal-free ligation, labeling, and surface derivatization, *Pure and Applied Chemistry*, ***85***(7), 1499-1513.

[5] **Sletten, E. M. and C. R. Bertozzi.** (2009). Bioorthogonal Chemistry: Fishing for Selectivity in a Sea of Functionality, *Angewandte Chemie-International Edition*, ***48***(38), 6974-6998.

[6] **Binder, W. H. and R. Sachsenhofer.** (2008). 'Click' Chemistry in Polymer and Material Science: An Update, *Macromolecular Rapid Communications*, ***29***(12-13), 952-981.

[7] **Devaraj, N. K., R. Weissleder , and S. A. Hilderbrand.** (2008). Tetrazine-Based Cycloadditions: Application to Pretargeted Live Cell Imaging, *Bioconjugate Chemistry*, ***19***(12), 2297-2299.

[8] **Chen, G., L. Tao, G. Mantovani, J. Geng, et al.** (2007). A Modular Click Approach to Glycosylated Polymeric Beads: Design, Synthesis and Preliminary Lectin Recognition Studies, *Macromolecules*, ***40***(21), 7513-7520.

[9] **Li, M., K. G. Neoh, L. Q. Xu, R. Wang, et al.** (2012). Surface Modification of Silicone for Biomedical Applications Requiring Long-Term Antibacterial, Antifouling, and Hemocompatible Properties, *Langmuir*, ***28***(47), 16408-16422.

[10] **He, F., B. Luo, S. Yuan, B. Liang, et al.** (2014). PVDF film tethered with RGD-click-poly(glycidyl methacrylate) brushes by combination of direct surface-initiated ATRP and click chemistry for improved cytocompatibility, *RSC Advances*, ***4***(1), 105-117.

[11] **Buhl, M., B. Vonhören , and B. J. Ravoo.** (2015). Immobilization of Enzymes via Microcontact Printing and Thiol–Ene Click Chemistry, *Bioconjugate Chemistry*, ***26***(6), 1017-1020.

[12] **Li, Y. and C. Cai.** (2011). Click Chemistry-Based Functionalization on Non-Oxidized Silicon Substrates, *Chemistry – An Asian Journal*, ***6***(10), 2592-2605.

[13] **Xue, C.-H., X.-J. Guo, M.-M. Zhang, J.-Z. Ma, et al.** (2015). Fabrication of robust superhydrophobic surfaces by modification of chemically

roughened fibers via thiol–ene click chemistry, *Journal of Materials Chemistry A*, ***3***(43), 21797-21804.

[14] **Feng, W., L. Li, E. Ueda, J. Li, et al.** (2014). Surface Patterning via Thiol-Yne Click Chemistry: An Extremely Fast and Versatile Approach to Superhydrophilic-Superhydrophobic Micropatterns, *Advanced Materials Interfaces*, ***1***(7), 1400269.

[15] **Gu, L., Q. Xue, S. Peng, G. Wang, et al.** (2016). A novel and facile strategy to inhibit corrosion: thiol-click synthesis of polythiols and their skinning on a metal surface to form super thick protective films, *Polymer Chemistry*, ***7***(3), 625-632.

[16] **Biggs, C. I., M. Walker , and M. I. Gibson.** (2016). "Grafting to" of RAFTed Responsive Polymers to Glass Substrates by Thiol–Ene and Critical Comparison to Thiol–Gold Coupling, *Biomacromolecules*, ***17***(8), 2626-2633.

[17] **Dietrich, M., G. Delaittre, J. P. Blinco, A. J. Inglis, et al.** (2012). Photoclickable Surfaces for Profluorescent Covalent Polymer Coatings, *Advanced Functional Materials*, ***22***(2), 304-312.

[18] **Doberenz, F., K. Zeng, C. Willems, K. Zhang, et al.** (2020). Thermoresponsive polymers and their biomedical application in tissue engineering - a review, *Journal of Materials Chemistry B*, ***8***(4), 607-628.

[19] **Grover, G. N. and H. D. Maynard.** (2010). Protein–polymer conjugates: synthetic approaches by controlled radical polymerizations and interesting applications, *Curr. Opin. Chem. Biol.*, ***14***(6), 818.

[20] **Pelegri-O'Day, E. M., E. W. Lin , and H. D. Maynard.** (2014). Therapeutic Protein–Polymer Conjugates: Advancing Beyond PEGylation, *J. Am. Chem. Soc.*, ***136***(41), 14323.

[21] **Liu, H., J. Bolleddula, A. Nichols, L. Tang, et al.** (2020). Metabolism of bioconjugate therapeutics: why, when, and how?, *Drug Metabolism Reviews*.

[22] **Jiang, Y. Y. and M. Stenzel.** (2016). Drug Delivery Vehicles Based on Albumin-Polymer Conjugates, *Macromol. Biosci.*, ***16***(6), 791.

[23] **Messina, M. S., K. M. M. Messina, A. Bhattacharya, H. R. Montgomery, et al.** (2020). Preparation of biomolecule-polymer conjugates by grafting-from using ATRP, RAFT, or ROMP, *Progress in Polymer Science*, ***100***, 25.

[24] **Tasdelen, M. A. and Y. Yagci.** (2013). Light-Induced Click Reactions, *Angewandte Chemie-International Edition*, ***52***(23), 5930-5938.

[25] **Tasdelen, M. A., B. Kiskan , and Y. Yagci.** (2016). Externally stimulated click reactions for macromolecular syntheses, *Progress in Polymer Science*, ***52***, 19-78.

[26] **Moad, G., E. Rizzardo , and S. H. Thang.** (2013). RAFT Polymerization and Some of its Applications, *Chem. - Asian J.*, ***8***(8), 1634.

[27] **Zhang, H., W. S. Trout, S. Liu, G. A. Andrade, et al.** (2016). Rapid Bioorthogonal Chemistry Turn-on through Enzymatic or Long

Wavelength Photocatalytic Activation of Tetrazine Ligation, *Journal of the American Chemical Society*, ***138***(18), 5978-5983.

[28] **Selvaraj, R. and J. M. Fox.** (2014). An efficient and mild oxidant for the synthesis of s-tetrazines, *Tetrahedron Letters*, ***55***(34), 4795-4797.

[29] **Truong, V. X., K. M. Tsang, F. Ercole , and J. S. Forsythe.** (2017). Red Light Activation of Tetrazine–Norbornene Conjugation for Bioorthogonal Polymer Cross-Linking across Tissue, *Chemistry of Materials*, ***29***(8), 3678-3685.

[30] **Zhang, H. L., J. Weingart, V. Gruzdys , and X. L. Sun.** (2016). Synthesis of an End-to-End Protein Glycopolymer Conjugate via Bio-Orthogonal Chemistry, *ACS Macro Lett.*, ***5***(1), 78.

[31] **Oliveira, B. L., Z. Guo , and G. J. L. Bernardes.** (2017). Inverse electron demand Diels–Alder reactions in chemical biology, *Chemical Society Reviews*, ***46***(16), 4895-4950.

[32] **Blackman, M. L., M. Royzen , and J. M. Fox.** (2008). Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels−Alder Reactivity, *Journal of the American Chemical Society*, ***130***(41), 13518-13519.

[33] **Devaraj, N. K., R. Upadhyay, J. B. Hatin, S. A. Hilderbrand, et al.** (2009). Fast and Sensitive Pretargeted Labeling of Cancer Cells through a Tetrazine/trans-Cyclooctene Cycloaddition, *Angewandte Chemie-International Edition*, ***48***(38), 7013-7016.

[34] **Devaraj, N. K. and R. Weissleder.** (2011). Biomedical Applications of Tetrazine Cycloadditions, *Accounts of Chemical Research*, ***44***(9), 816-827.

[35] **Liu, D. S., A. Tangpeerachaikul, R. Selvaraj, M. T. Taylor, et al.** (2012). Diels–Alder Cycloaddition for Fluorophore Targeting to Specific Proteins inside Living Cells, *Journal of the American Chemical Society*, ***134***(2), 792-795.

[36] **Koo, H., M. Choi, E. Kim, S. K. Hahn, et al.** (2015). Bioorthogonal Click Chemistry-Based Synthetic Cell Glue, *Small*, ***11***(48), 6458-6466.

[37] **Šečkutė, J. and N. K. Devaraj.** (2013). Expanding room for tetrazine ligations in the in vivo chemistry toolbox, *Current Opinion in Chemical Biology*, ***17***(5), 761-767.

[38] **Zhang, C. J., C. Y. J. Tan, J. Ge, Z. Na, et al.** (2014). Preparation of Small-Molecule Microarrays by trans-Cyclooctene Tetrazine Ligation and Their Application in the High-Throughput Screening of Protein–Protein Interaction Inhibitors of Bromodomains, *Angewandte Chemie International Edition*, ***52***(52), 14060-14064.

[39] **Alge, D. L., M. A. Azagarsamy, D. F. Donohue , and K. S. Anseth.** (2013). Synthetically Tractable Click Hydrogels for Three-Dimensional Cell Culture Formed Using Tetrazine–Norbornene Chemistry, *Biomacromolecules*, ***14***(4), 949-953.

[40] **Hansell, C. F., P. Espeel, M. M. Stamenović, I. A. Barker, et al.** (2011). Additive-Free Clicking for Polymer Functionalization and Coupling by

Tetrazine–Norbornene Chemistry, *Journal of the American Chemical Society*, ***133***(35), 13828-13831.

[41] **Url-1**<*http://www.clickchemistrytools.com*>, date retrieved 29.05.2020.

[42] **Mayer, S. V., A. Murnauer, M.-K. von Wrisberg, M.-L. Jokisch, et al.** (2019). Photo-induced and Rapid Labeling of Tetrazine-Bearing Proteins via Cyclopropenone-Caged Bicyclononynes, *Angewandte Chemie International Edition*, ***58***(44), 15876-15882.

[43] **Jiang, T., P. Kumar, W. Huang, W.-S. Kao, et al.** (2019). Modular Enzyme- and Light-Based Activation of Cyclopropene–Tetrazine Ligation, *ChemBioChem*, ***20***(17), 2222-2226.

[44] **Zhang, H., W. S. Trout, S. Liu, G. A. Andrade, et al.** (2016). Rapid Bioorthogonal Chemistry Turn-on through Enzymatic or Long Wavelength Photocatalytic Activation of Tetrazine Ligation, *Journal of the American Chemical Society*, ***138***(18), 5978-5983.

[45] **Yang, J., M. R. Karver, W. L. Li, S. Sahu, et al.** (2012). Metal-Catalyzed One-Pot Synthesis of Tetrazines Directly from Aliphatic Nitriles and Hydrazine, *Angewandte Chemie-International Edition*, ***51***(21), 5222-5225.

[46] **Keddie, D. J., G. Moad, E. Rizzardo , and S. H. Thang.** (2012). RAFT Agent Design and Synthesis, *Macromolecules*, ***45***(13), 5321-5342.

[47] **McCormick, C. L. and A. B. Lowe.** (2004). Aqueous RAFT Polymerization: Recent Developments in Synthesis of Functional Water-Soluble (Co)polymers with Controlled Structures, *Accounts of Chemical Research*, ***37***(5), 312-325.

[48] **Moad, G., Y. K. Chong, A. Postma, E. Rizzardo, et al.** (2005). Advances in RAFT polymerization: the synthesis of polymers with defined end-groups, *Polymer*, ***46***(19), 8458-8468.

[49] **Moad, G., E. Rizzardo , and S. H. Thang.** (2009). Living Radical Polymerization by the RAFT Process - A Second Update, *Aust. J. Chem.*, ***62***(11), 1402.

[50] **Schild, H. G.** (1992). Poly(N-isopropylacrylamide): experiment, theory and application, *Progress in Polymer Science*, ***17***(2), 163-249.

[51] **Chung, J. E., M. Yokoyama, M. Yamato, T. Aoyagi, et al.** (1999). Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly(N-isopropylacrylamide) and poly(butylmethacrylate), *Journal of Controlled Release*, ***62***(1), 115-127.

[52] **Lee, E. L. and H. A. von Recum.** (2010). Cell culture platform with mechanical conditioning and nondamaging cellular detachment, *Journal of Biomedical Materials Research Part A*, ***93A***(2), 411-418.

[53] **Nesrinne, S. and A. Djamel.** (2017). Synthesis, characterization and rheological behavior of pH sensitive poly(acrylamide-co-acrylic acid) hydrogels, *Arabian Journal of Chemistry*, ***10***(4), 539-547.

[54] **Huffman, A. S., A. Afrassiabi , and L. C. Dong.** (1986). Thermally reversible hydrogels: II. Delivery and selective removal of substances from aqueous solutions, *Journal of Controlled Release*, ***4***(3), 213-222.

[55] **Okano, T., Y. H. Bae, H. Jacobs , and S. W. Kim.** (1990). Thermally on-off switching polymers for drug permeation and release, *Journal of Controlled Release*, ***11***(1), 255-265.

[56] **Lambeth, R. H., S. Ramakrishnan, R. Mueller, J. P. Poziemski, et al.** (2006). Synthesis and aggregation behavior of thermally responsive star polymers, *Langmuir*, ***22***(14), 6352-6360.

[57] **Yu, B., J. W. Chan, C. E. Hoyle , and A. B. Lowe.** (2009). Sequential Thiol-Ene/Thiol-Ene and Thiol-Ene/Thiol-Yne Reactions as a Route to Well-Defined Mono and Bis End-Functionalized Poly(N-isopropylacrylamide), *Journal of Polymer Science Part a-Polymer Chemistry*, ***47***(14), 3544-3557.

[58] **Roth, P. J.** (2014). Composing Well-Defined Stimulus-Responsive Materials Through Postpolymerization Modification Reactions, *Macromolecular Chemistry and Physics*, ***215***(9), 825-838.

[59] **Chong, Y. K., G. Moad, E. Rizzardo , and S. H. Thang.** (2007). Thiocarbonylthio End Group Removal from RAFT-Synthesized Polymers by Radical-Induced Reduction, *Macromolecules*, ***40***(13), 4446-4455.

[60] **Matyjaszewski, K.** (2020). Discovery of the RAFT Process and Its Impact on Radical Polymerization, *Macromolecules*, ***53***(2), 495-497.

[61] **Willcock, H. and R. K. O'Reilly.** (2010). End group removal and modification of RAFT polymers, *Polymer Chemistry*, ***1***(2), 149-157.

[62] **Boyer, C., V. Bulmus, T. P. Davis, V. Ladmiral, et al.** (2009). Bioapplications of RAFT Polymerization, *Chem. Rev. (Washington, DC, U. S.)*, ***109***(11), 5402.

[63] **Inglis, A. J., S. Sinnwell, T. P. Davis, C. Barner-Kowollik, et al.** (2008). Reversible Addition Fragmentation Chain Transfer (RAFT) and Hetero-Diels−Alder Chemistry as a Convenient Conjugation Tool for Access to Complex Macromolecular Designs, *Macromolecules*, ***41***(12), 4120-4126.

[64] **Gody, G., C. Rossner, J. Moraes, P. Vana, et al.** (2012). One-Pot RAFT/“Click” Chemistry via Isocyanates: Efficient Synthesis of α-End-Functionalized Polymers, *Journal of the American Chemical Society*, ***134***(30), 12596-12603.

[65] **Bertazzo, S., W. Zambuzzi, H. Silva, C. Ferreira, et al.** (2009). Bioactivation of alumina by surface modification: A possibility for improving the applicability of alumina in bone and oral repair, *Clinical oral implants research*, ***20***, 288-93.

[66] **Pulker, H. K.** (1984). *Coatings on glass*, Amsterdam, Elsevier.

[67] **Sugarman, B.** (1967). Strength of glass (a review), *Journal of Materials Science*, ***2***(3), 275-283.

[68] **Taga, Y.** (1997). Recent progress in coating technology for surface modification of automotive glass, *Journal of Non-Crystalline Solids*, ***218***, 335-341.

[69] **Liu, A. P., A. M. Streltsov, X. H. Li , and A. A. Abramov,** (2014). Laser Processing of Glass for Consumer Electronics: Opportunities and Challenges, *Laser Processing and Fabrication for Solar, Displays, and Optoelectronic Devices ıı, The International optics and photonics Conference,* San Diego, California, United States.

[70] **Park, S., J. Pai, E.-H. Han, C.-H. Jun, et al.** (2010). One-Step, Acid-Mediated Method for Modification of Glass Surfaces with N-Hydroxysuccinimide Esters and Its Application to the Construction of Microarrays for Studies of Biomolecular Interactions, *Bioconjugate Chemistry*, ***21***(7), 1246-1253.

[71] **Kim, T. H., R. K. Singh, M. S. Kang, J. H. Kim, et al.** (2016). Gene delivery nanocarriers of bioactive glass with unique potential to load BMP2 plasmid DNA and to internalize into mesenchymal stem cells for osteogenesis and bone regeneration, *Nanoscale*, ***8***(15), 8300-8311.

[72] **Kotsuchibashi, Y. Nakagawa , and Ebara**, **M.** (2016). Biomaterials Nanoarchitectonics. *Nanoparticles* (Vol. 1, pp.7-23).Retrieved from http://www.sciencedirect.com/science/article/pii/B9780323371278000029

[73] **Wang, J. S. and K. Matyjaszewski.** (1995). Controlled/″living″ radical polymerization. atom transfer radical polymerization in the presence of transition-metal complexes, *J. Am. Chem. Soc.*, ***117***(20), 5614.

[74] **Kulkarni, S., C. Schilli, A. H. E. Müller, A. S. Hoffman, et al.** (2004). Reversible meso-scale smart polymer-protein particles of controlled sizes, *Bioconjugate Chemistry*, ***15***(4), 747-753.

[75] **Vig, B. S., K. M. Huttunen, K. Laine , and J. Rautio.** (2013). Amino acids as promoieties in prodrug design and development, *Advanced Drug Delivery Reviews*, ***65***(10), 1370-1385.

[76] **Kara, S. S., M. Y. Ateş, G. Deveci, A. Cetinkaya, et al.** (2019). Direct synthesis of tetrazine functionalities on polymer backbones, *Journal of Polymer Science Part A: Polymer Chemistry*, ***57***(6), 673-680.

[77] **Ferguson, C. J., R. J. Hughes, D. Nguyen, B. T. T. Pham, et al.** (2005). Ab Initio Emulsion Polymerization by RAFT-Controlled Self-Assembly, *Macromolecules*, ***38***(6), 2191-2204.

[78] **Royzen, M., G. P. A. Yap , and J. M. Fox.** (2008). A photochemical synthesis of functionalized trans-cyclooctenes driven by metal complexation, *Journal of the American Chemical Society*, ***130***(12), 3760-3761.

[79] **Biggs, C. I., M. Walker , and M. I. Gibson.** (2016). "Grafting to" of RAFTed Responsive Polymers to Glass Substrates by Thiol-Ene and Critical Comparison to Thiol-Gold Coupling, *Biomacromolecules*, ***17***(8), 2626-2633.

[80] **Hermanson, Greg T.** (2008). Chapter 13-Silane Coupling Agents , *Bioconjugate Technique*, (Vol.2, pp.562-581) Retrieved from http:// www.sciencedirect.com/ science/article/pii/ B9780123705013000138

[81] **Galagan, Y. and W.-F. Su.** (2008). Reversible photoreduction of methylene blue in acrylate media containing benzyl dimethyl ketal, *Journal of Photochemistry and Photobiology A: Chemistry*, ***195***(2), 378-383.

[82] **Alghunaim, A., E. T. Brink , and B.-M. Z. Newby.** (2016). Surface immobilization of thermo-responsive poly(N-isopropylacrylamide) by simple entrapment in a 3-aminopropyltriethoxysilane network, *Polymer*, ***101***, 139-150.

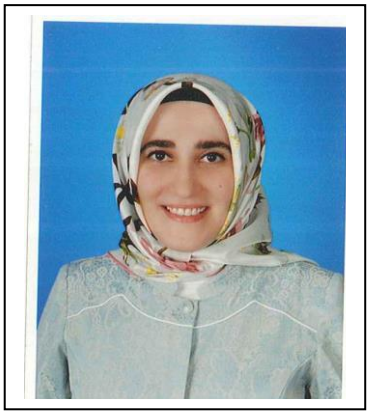
[83] **Dong, Y., X. Zhu, F. Shi , and J. Nie.** (2014). Surface photo-anchored PNIPAM crosslinked membrane on glass substrate by covalent bonds, *Applied Surface Science*, ***307***, 7-12.

[84] **Khalifehzadeh, R. and B. D. Ratner.** (2019). Trifluoromethyl-functionalized poly(lactic acid): a fluoropolyester designed for blood contact applications, *Biomaterials Science*, ***7***(9), 3764-3778.

[85] **Fan, X., Y. Ge, F. Lin, Y. Yang, et al.** (2016). Optimized tetrazine derivatives for rapid bioorthogonal decaging in living cells, *Angew Chem International Edition*, ***55***(45), 14046-14050.

[86] **Guo, X., S. Sang, A. Jian, S. Gao, et al.** (2018). A bovine serum albumin-coated magnetoelastic biosensor for the wireless detection of heavy metal ions, *Sensors and Actuators B: Chemical*, ***256***, 318-324.

CURRICULUM VITAE



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- **Ozbek, N., Kahveci U.M.** 2019: Preparation of Functional Surfaces with Light-induced Tetrazine Click Reaction. East West Chemistry Conference 2019, November 13-15, 2019 Palermo, Italy.