

T.R.
GEBZE TECHNICAL UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY

**PREPARATION OF PHOTSENSITIVE HYDROGELS BY
BLUE LIGHT-INDUCED PHOTOPOLYMERIZATION AND
FORMATION OF BIO-FUNCTIONALIZABLE MICROWELLS
BY UV-MEDIATED ETCHING**

MEHMET KIRANŞAL
A THESIS SUBMITTED FOR THE DEGREE OF
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ASSIST. PROF. TUĞÇE NİHAL GEVREK CİVAN

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2023

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
BİYOTEKNOLOJİ ENSTİTÜSÜ

MAVİ IŞIK KULLANILARAK
FOTOPOLİMERİZASYON YOLUYLA IŞIĞA
DUYARLI HİDROJELLERİN
HAZIRLANMASI VE UV ARACILI
AŞINDIRMA İLE BİYO-
İŞLEVSELLEŞTİRİLEBİLİR
MİKROKUYULARIN OLUŞTURULMASI

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GEBZE
2023



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SUMMARY

Light can cause isomerization, dimerization, or cleavage of photosensitive groups due to the presence of photochromic units in photosensitive materials, which capture the optical signal and converts it into a chemical transformation through a photoreaction. To undertake these processes, the presence of photosensitive groups in the hydrogel networks are incorporated. One of the commonly used photosensitive groups in literature is the ortho-nitrobenzyl ester (ONB) group due to its facile cleavage upon UV irradiation (365 nm) and its suitability for biological applications. It is established that the decomposition of the nitrobenzyl group in the presence of UV results in a nitrosobenzaldehyde group which has reactivity toward amine-bearing molecules.

Herein, poly(ethylene glycol) methyl ether methacrylate (PEG)-based hydrogels containing light-responsive ONB in the crosslinking points were prepared on glass surfaces using photopolymerization under visible blue light, which does not decompose the ortho nitrobenzyl ester units. Four different hydrogels were obtained by keeping the amount of PEG constant and varying the ratio of the ONB-containing crosslinker and PEGDA. Hydrogels were then exposed to UV light through a photomask to decompose ONB at crosslinker sites to yield an amine-reactive nitroso benzaldehyde unit containing micro-patterns. To observe the reactive groups and microwells formations, FTIR spectroscopy and SEM analyses were performed respectively. It was shown that the density of reactive groups in microwells can be controlled by adjusting the ratio of the UV-responsive ONB-based crosslinker in hydrogels. Additionally, efficient (bio) functionalization in the fabricated microwells could be achieved through photogenerated nitrosobenzaldehyde and monitored by a fluorescent microscope.

Keywords: Photopolymerization, orthonitrobenzaldehyde, UV-etching, nitroso benzaldehyde, microwells, biofunctionalization.

ÖZET

Işık, ışığa duyarlı malzemelerdeki fotokromik birimlerin optik sinyali yakalayıp ve bunu bir foto reaksiyon yoluyla kimyasal bir sinyale dönüştürerek izomerleşmesine, dimerleşmesine veya ışığa duyarlı grupların bölünmesine neden olabilir. Bu değişiklikler, hidrojel yapısının şişmesine, büzülmesine ve/veya kısmen veya tamamen bozulmasına yol açabilirler. Bu işlemlerin gerçekleştirilebilmesi için hidrojel ağlarında ışığa duyarlı gruplar yerleştirilmektedir. Literatürde yaygın olarak kullanılan ışığa duyarlı grup, UV ışık altında (365 nm) kolayca parçalanabilmesi ve biyolojik uygulamalar için yüksek duyarlılığı nedeniyle orto-nitrobenzil ester (ONB) grubudur. UV varlığında nitrobenzil gruplarının parçalanarak aminlere karşı reaktivitesi olan nitrozobenzaldehit grubu vermesi literatürde gösterilmiştir.

Bu çalışmada, orto nitrobenzil birimlerinde parçalanmaya yol açmayan görünür mavi ışık altında fotopolimerizasyonu yoluyla cam üzerinde ışığa duyarlı ONB içeren Poli(etilen glikol)metil eter metakrilat) (PEG) bazlı hidrojeller hazırlanmıştır. PEG oranı sabit tutulup, ONB ester içeren çapraz bağlayıcıyla PEGDA'nın birbirine oranı değiştirilmesi ile dört farklı hidrojel elde edilmiştir. Hidrojeller daha sonra, mikrokuyu içeren amin reaktif nitrozo benzaldehit birimlerini vermek üzere çapraz bağlayıcı bölgelerindeki ONB'yi ayrıştırmak için fotomaske arkasından UV ışığına maruz bırakılmıştır. Reaktif grupların ve mikrokuyuların oluşumu FTIR ve SEM ile karakterize edilmiştir. Mikrokuyulardaki reaktif grup yoğunluğunun, hidrojellerdeki UV'ye duyarlı çapraz bağlayıcı oranı değiştirilerek kontrol edilebileceği gösterilmiştir. Ayrıca, elde edilen mikrokuyuların ışık etkisiyle ortaya çıkan nitrozobenzaldehit aracılığıyla etkin bir şekilde (biyo)işlevselleştirilmesi başarıyla gerçekleştirilmiş ve floresan mikroskop ile takip edilmiştir.

Anahtar kelimeler: Fotopolimerizasyon, ortonitrobenzaldehit, UV-aşındırma, nitrozobenzaldehit, mikro kuyucuklar, biyofonksiyonelleştirme

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LIST OF ABBREVIATIONS and ACRONYMS

Abbreviations and

Acronyms

Explanations

DEGMEMA	: Di(ethylene glycol) methyl ether methacrylate
DMF	: Dimethylformamide
FTIR	: Fourier Transform Infrared Spectroscopy
NTPA	: Nitroterephthalic acid
NPDM	: (2-Nitro-1,4-phenylene) dimethanol
NPDMA	: (2-Nitro-1,4-phenylene) bis(methylene) diacrylate
ONB	: orthonitrobenzyl
PEGMEMA	: Polyethylene Glycol Methyl Ethyl Methacrylate
SEM	: Scanning Electron Microscope
TEA	: Triethylamine
THF	: Tetrahydrofuran
TPA	: Terephthalic acid

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1. INTRODUCTION

Hydrogels are defined as three-dimensional (3D), water-insoluble, water-swelling crosslinked polymers. A hydrogel structure maintains its stability by specific chemical, or physical crosslinking of individual polymer chains. Hydrogel scaffolds are generally composed of hydrophilic polymer chains. Presence of specific functional groups such as $-NH_2$, $-SO_3H$, $-COOH$, $-CONH_2$, $-OH$, provide this hydrophilic feature to hydrogels [1]. Water molecules interact with these functional groups, and thus hydrogels can retain high amount of water due to these hydrophilic chains [1]. In order to be defined a polymer as ‘hydrogel’, water must constitute at least 10% of total weight [1]. Hydrogels are also flexible, soft, and porous materials. Such features enable hydrogels to mimic the extracellular matrix, and thus have attracted widespread attention of scientists [2].

The term as ‘hydrogel’ was introduced with literature by von Bemmelen to explain colloidal gels in 1894. However, first biotechnologic study of hydrogels was carried out by Wichterle and Lim in 1960 [3]. They described a crosslinked hydrogel based on 2- hydroxyethyl methacrylate (HEMA) to design contact lenses. This marked a significant milestone in the utilization of hydrogels as biomaterials. [3].

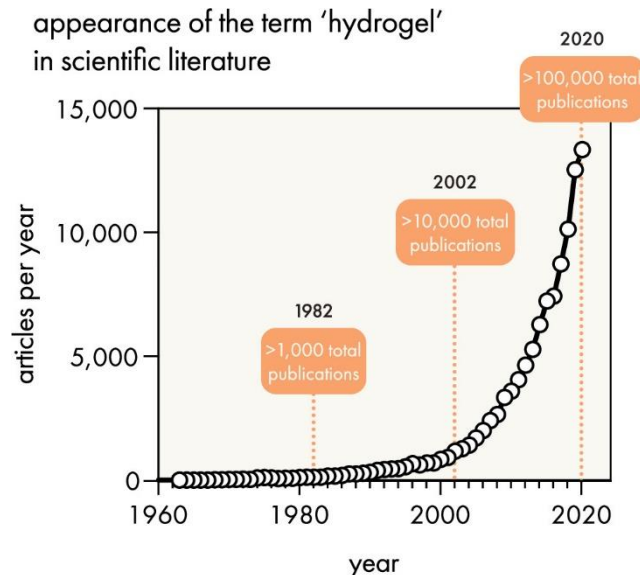


Figure 1: Publications referencing ‘hydrogels’ over years 1960-2020 [4].

After 1960, hydrogel applications gradually increased. However, over the next two decades, hydrogel applications and research relatively remained on simple crosslinked polymers, focusing on ophthalmic and drug delivery systems [4]. One example of a hydrogel used in biotechnological applications is the micro-capsular membrane designed by Lim and Sun in 1980. The membrane was made up of cross-linked alginate molecules and was intended for use in cell engineering systems. [5]. Yannas and Gordon subsequently described a new biomaterial based on natural polymer collagen. As illustrated in Figure 1, there were around 1000 publications by 1980. However, by 2020, the total number of publications related to hydrogels had soared to 100,000. This trend indicates a continually growing interest in these materials due to their potential applications across various areas of biomedical sciences.

In the beginning, hydrogel studies started with simple crosslinked polymers designed for contact lens and drug delivery applications. As developments in organic and polymer chemistry progressed, other natural and synthetic polymers were also used as hydrogel scaffolds. There were several well-known polymers such as poly(ethylene glycol) (PEG) and poly(vinyl alcohol) (PVA), widely used in early 80s and 90s [5]. Understanding the mechanisms of polymerization technologies allows researchers to develop more complicated hydrogel systems. The limited usage and application areas of hydrogel systems have expanded due to the development in material fabrication over the years.

1.1. Applications of Hydrogels

As mentioned earlier, hydrogels are flexible and soft materials, with their flexibility being provided by their water content. In addition to these unique features, hydrogels are known for being non-toxic and biocompatible nature. Their porous and 3D network structure allows for the placement of cells and other biomolecules [6]. Crosslinkers contribute to the formation of a stable structure that resists dissolution in high amounts of water content. They also enhance the durability of hydrogels against environmental effect. [7]. Overall, advancements in hydrogel research, as well as the development of new monomer and polymer systems, have not only made hydrogels valuable in drug delivery systems but have also expanded their

applications across various fields, ranging from industrial to medical biotechnology applications [8]. The following sections enlist and briefly explain several of these applications (Figure 1.2).

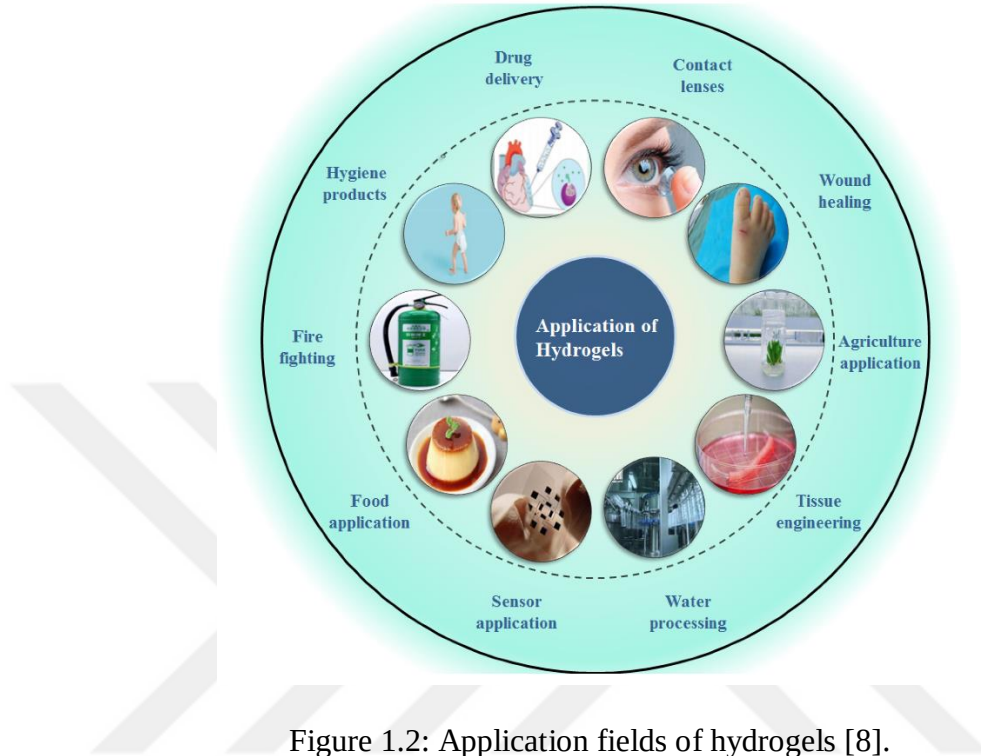


Figure 1.2: Application fields of hydrogels [8].

1.1.1. Drug Delivery Systems

One of the features that make hydrogels as attractive scaffolds is their porous structure. Porosity of hydrogels is generally determined by two factors: crosslinked level of matrix and affinity of hydrogel to environment with respect to swollen state [7]. Another important parameter is the mesh size which means the gap between two adjacent polymer chains connected by a crosslinker [7]. The presence of porous structures and the ability to adjust the mesh size allow for precise control over the loading and release of desired drug molecules. As a result, hydrogels are increasingly garnering attention as promising drug delivery systems [9]. Hydrogels offer several advantages as a drug delivery system. Firstly, they provide controlled release of drugs. Secondly, the protection of the hydrogel scaffold can prevent the degradation of drug molecules. [10].

There are two mechanisms for drug release from hydrogels: diffusion-controlled and swelling-controlled release. In swelling-based systems, drug

molecules are dispersed throughout the polymer matrix. When the polymer encounters biological fluid, it starts to swell, allowing the drugs to pass from the polymer matrix into the biological environment. Lastly, chemically controlled release is based on environmental factors such as hydrolytic events, enzymatic activity from biological content, and cleavage of the polymer structure, which cause the release of drug molecules from the gels [11].

1.1.2. Tissue Engineering Applications as Scaffold Systems

Tissue engineering is a set of engineering techniques and applications to produce or repair the body's damaged or injured functions [12]. Applications in this context include repairing of any organ tissue, delivery of chemical or biological drugs, and design of artificial organs and tissues. Cancer and related cell-based applications are also grouped under cell cultivation systems [12].

Tissue engineering applications of hydrogels hold promising potential for the future. One of the most important advantages of hydrogels is their ability to mimic the extracellular matrix. In traditional cell cultures used for drug development and tissue regeneration applications, cells are typically grown in 2D environments, which can limit drug efficiencies. Also, 2D cell cultures also limit normal cell growth and may affect the overall results [13].

Hydrogels have demonstrated biocompatibility and non-toxicity, rendering them suitable for supporting cell growth. Their porous structures facilitate the appropriate placement and growth of cells within the gel matrix. Additionally, hydrogels mimic the stiffness of natural tissues, allowing cells within the gel scaffolds to maintain their native state and preserve their morphology. As a result, the molecular mechanisms occurring in the cytoplasm and nucleus of these cells closely resemble native cell behavior. [14]. As mentioned, that hydrogels provide a 3D environment and enable cells to undergo cell-cell and cell-environment interaction like in an *in vivo* setting [14].

1.1.3. Contact Lenses

In 1960, a first-generation hydrogel used as contact lenses which was developed by Wichterle and Lim opened a new era for both contact lenses and hydrogel applications. This simple polymer structure was composed of poly-2-hydroxymethacrylate (pHEMA) [15]. This report opened the application of hydrogels as soft contact lenses in real life. pHEMA lenses were distributed across Europe two years later. In 1965, National Patent Development Corporation in US bought licence of pHEMA based contact lenses [16].

In order to design a material that will be used as a contact lens, it must possess several properties such as oxygen permeability, proper mechanical properties and wettability. When contact lenses are directly placed on the cornea, they can impede the exchange of atmospheric oxygen to corneal cells. This interference with gas exchange can disrupt cellular mechanisms, hinder healthy maintenance, and ultimately cause hypoxic stress. Therefore, a polymer scaffold used for contact lenses must have maximum oxygen permeability [16]. Polymer structures show also proper mechanic features to prevent mechanic stress. Moreover, hydrogel systems should be wettable and nontoxic for tolerance of cell living, having good optical facilities. Hydrogel scaffolds should be stable and resistant against hydrolytic cleavage and enzymatic activities.

Over the years, from first contact lens application in 1960, there have been numerous advancements in contact lens technology. Researchers have discovered and utilized appropriate monomers to adjust the toxicity levels and improve the wettable conditions of the lens scaffold. [16].

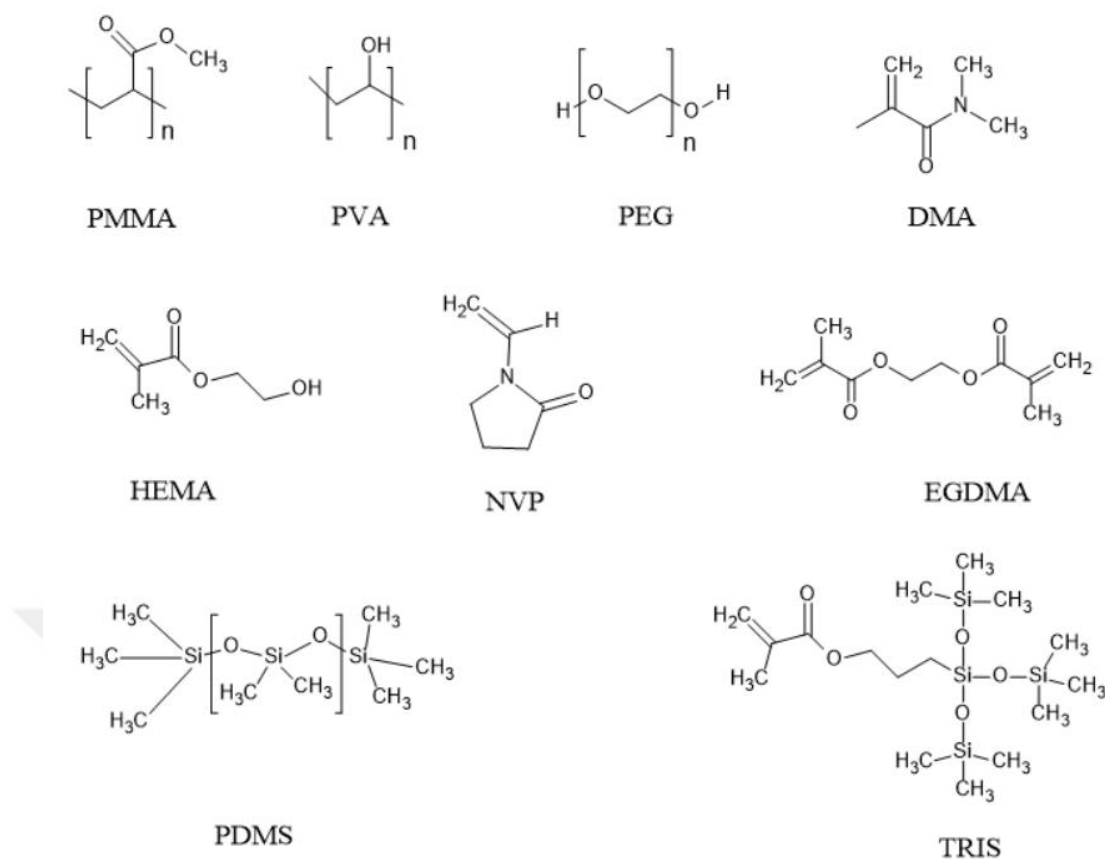


Figure 1.3: Common monomers used in contact lenses [16].

1.1.4. Biosensor Applications

A biosensor is an analytic device that measures a biological or chemical reactions by generating response which is proportional to concentration of an analyte of interest in a medium [17]. A biosensor is generally comprised of three main parts; sensor segment that which is made up of biological components, detects the analyte, second as transducer which convert biologic response into measurable signal [18]. Final part is the data processing site which gives meaningful measurement about analyte [18]. Hydrogel matrices are generally used in two ways for biosensor applications. The first way is using hydrogels without biologic elements. In this approach, the hydrogel matrix itself can respond to changes in biologic analytes, showing variations in swelling, pH, temperature sensitivity, and even electrical properties. The second one is using hydrogel matrices as scaffolds for immobilizing biological elements. This approach increases the specificity of the sensor and ensures the stability of the biorecognition probe [19].

1.2. Classifications of Hydrogels

Hydrogels can be classified according to different features and characteristics. For example, to construct a scaffold, monomers can be chosen either by natural, synthetic or hybrid. Polymer chains can be homopolymer, copolymer or multipolymers that is combination of two or more polymer chains. Monomers are crosslinked by either physical or chemical methods. Polymer chains can have neutral, ionic, ampholytic, or zwitterionic charges. Hydrogels can also be classified based on their response, which can be physical, chemical, or biochemical. Additionally, they can exist in amorphous, crystalline, or semicrystalline configurations (Figure 1.4). [20]. [21]



Figure 1.4: Classifications of hydrogels [22].

1.2.1. Based on Sources

Hydrogels are divided into three sections according to source which are natural, synthetic or hybrid. Natural polymers are obtained from natural sources such as animals, plants or algae. It can be generally grouped under proteins (collagen, fibrinogen, gelatin) or polysaccharides (cellulose, starch, chitosan, hyaluronic acid, dextran, alginate.) [21]. Natural-based hydrogels offer several advantages. Since they are derived from natural sources, they are highly biocompatible, biodegradable, and exhibit low toxicity for cells, which results in minimal immunogenic response. However, they also come with certain disadvantages. For instance, hydrogels based on natural materials can be vulnerable to contamination by viruses or bacteria, and they often demonstrate relatively lower mechanical strength when compared to their synthetic counterparts. According to a literature example in 2019, Park and coworkers developed a hydrogel scaffold for cartilage tissue engineering by using hyaluronic acid. In another study, alginate was used to produce hydrogel to be used in drug delivery vehicles, wound dressing, and 3D bioprinting [23].

On the other hand, synthetic hydrogels have several advantages. Firstly, they exhibit better mechanical properties, showing improved resistance against solvation. Moreover, they may have the ability to hold more water upon conjugation of proper units [24]. However, one of the biggest drawbacks of synthetic hydrogels is their limited immune response and the absence of cell-specific bioactivities. PEG polymers are frequently used in the literature due to unique advantages, such as good solubility, excellent biocompatibility, non-toxicity, and low immunogenicity.

Hybrid hydrogels are defined as combinations of two or more both natural and synthetic polymers. Their building blocks can be chemically, morphologically, and functionally distinct from each other. According to requirement, these polymers can be arranged and altered. Hybrid hydrogel scaffolds can have biologically active peptide blocks, proteins, or other nanoparticles in the structures. However, one of these polymers must be hydrophilic to sustain hydrophilic interactions for water absorption. As an example, Urello and coworkers designed a hydrogel structure that collagen mimetic peptide polyplex complex mediated growth factor delivery carried out by collagen gels [25].

1.2.2. Based on Chain Composition

Hydrogels are classified in three sections as homopolymeric, copolymeric and interpenetrating according chosen synthesis methods or crosslinker agents [26]. Homopolymers are polymer structures composed of only one type of monomer [27]. These monomer units are crosslinked by crosslinking agents to form a scaffold. Depending on the chosen synthesis strategy, polymerization takes place either with crosslinker agents or without them. However, such hydrogels may sometimes have disadvantages; a single monomer type cannot fulfill all the requirements, such as good swelling ability or proper mechanical features. [28].

On the other hand, copolymeric hydrogels are comprised of two different monomers. Therefore, they can present both natures of two polymers [29]. However, one these monomers should be hydrophilic for presenting swelling characteristic. According to configuration of monomer blocks or structures, copolymer is divided in block, graft, alternating or random types (Figure 1.5)

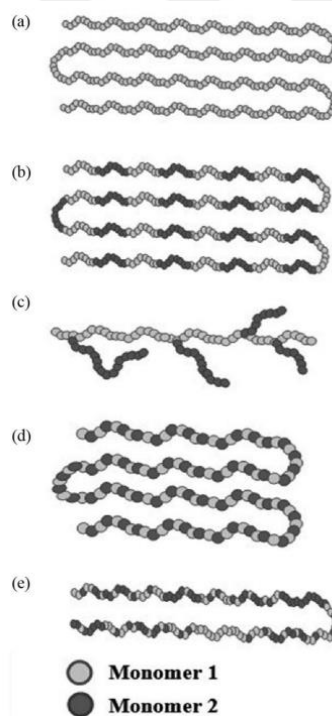


Figure 1.5: a) homopolymer, (b) Block copolymeric hydrogel, (c) Graft copolymeric hydrogel, (d) Alternating copolymeric hydrogel (e) Random type copolymeric hydrogel [30].

To produce copolymeric hydrogels, multifunctional crosslinkers are preferred and chemical polymerization methods are chosen in the presence of initiators.

Lastly, interpenetrating polymers (IPNs) are the hydrogel structures that consist of at least. There are no chemical linkages between two polymers, and they are not separated until chemical bonds are broken. Generally, one hydrophilic polymer is dropped into solvent that contains monomers of second polymers [28], [30]. Following the swelling of first polymer, polymerization of second polymers has completed. Due its network and characteristics of two polymers, interpenetrated hydrogels offer some advantageous, high order in stability, denser hydrogel matrix, improved mechanical properties like stiffness and toughness making them suitable drug loading scaffolds [28].

1.2.3. Based on Nature of Crosslinking

Crosslinking involves the formation of bonds or links (interactions) between two polymer chains. A polymer solution (gel precursor) transitions into a solid phase (gel) through crosslinking. As a result of crosslinking, polymers acquire increased molecular weight, improved mechanical strength, and heightened resistance to heat and solvents [31]. Arranging the crosslinking (amount of crosslinkers, crosslinker types) can alter the degree of swelling ratio, mechanical properties (softness, flexibility, elasticity) and release kinetics (porous/ mesh size alteration). There are basically 2 main crosslinking methods: physical or chemical [32]. Physical crosslinking is formed by Van der Waals interactions, ionic interactions, hydrogen bonding, hydrophobic interactions, and electrostatic interactions [32]. These kinds of hydrogels tend to be reversible (sol-gel transition) and gel formation can be altered by environmental factors (temperature, pH etc.) As its name suggests, constructing such hydrogel doesn't require the use of a crosslinker. Those hydrogels can be used in tissue engineering applications, wound healing, implantation and drug delivery systems. Chemical crosslinking occurs in the presence of crosslinking agents, resulting in the formation of polymer structures with irreversible configurations due to stable covalent bonds [33].

1.2.4. Based on Charges

Hydrogels are also classified according to charges they have. Such polymer structures are cationic, anionic, neutral or zwitterionic according to net charges [34]. Neutral polymers refer that net charge of total polymer structure is zero due to side groups or polymer backbones. As for cationic hydrogels carry positively charged polymer functional groups such as amines and thiols. Those types of hydrogels tend to show swelling ability in low pH. Anionic hydrogels have negatively charged functional groups on polymeric structures. Sulfonyl and carboxyl are the examples for the functional groups. Lastly, zwitterionic hydrogels contain both cationic and anionic groups inside the functional groups. This ability makes them suitable biomaterial for the biotechnologic applications [30], [35].

1.2.5. Based on Configuration

Hydrogels are also classified according to constitution of polymers that form the 3D structure. They can be in the form of amorphous, crystalline, or semi-crystalline. Amorphous hydrogels refer that polymer blocks that build up hydrogel scaffold are dispersed in space. They are placed randomly in the scaffold. They can be flexible and soft, due to its 3D shape they can be used as wound dressings and drug deliveries. However, crystalline hydrogels are formed in determined order. Crystalline hydrogels tend to be rigid and hard [36]. They show good mechanical properties and have a better capacity to resist against harsh conditions (high temperature, low or high pH etc.).

1.2.6. Based on External Stimuli Response

Hydrogels can also be classified according to external responses. Hydrogels may show swelling or shrinking behavior against some environmental conditions such as temperature, pH electrical or magnetic changes. Based on monomer units and synthesis methods, features of hydrogels are arranged. One of the most common applications of of such hydrogels are drug release. With the presence of external stimuli, drug release networks can be directed to specific areas. In such contexts,

controlled drug release occurs in response to slight environmental changes. Hydrogels that alter their physical or chemical properties in response to external stimuli are often referred to as smart hydrogels. [37].

1.3. Synthesis of Hydrogels

Hydrogels are prepared either by physical strategies or chemical strategies [6]. Physical strategies consist of heating or cooling of polymer solution, hydrophobic interactions, complex coacervation, ionic interactions, freeze thawing and hydrogen bonding [38]. On the other hands chemical strategies are divided into 2 main sections: Grafting and Crosslinking. Crosslinking strategies can be categorized as chemical crosslinking, radiation crosslinking, free radical cross-linking, and enzyme crosslinking. In radiation polymerization, there are different types of polymerization strategies [36], [38]. Photopolymerization is a subtype of radiation polymerization that uses visible or ultraviolet (UV) sources to start polymerization of monomers [39]. In physical strategies, there are various interactions between polymer blocks. However, these interactions are relatively weaker than chemically crosslinked bondings. For instance, ion interaction, hydrophobic interaction, hydrogen bonding and van der Waals interactions are preferred in physical crosslinking methods. Crosslinking agents are not needed for all physical crosslinking. Therefore, they show low toxicity and high biocompatibility. Absence of external crosslinkers also makes hydrogel scaffolds transition between gel-sol or sol-gel formations [40].

Although physical crosslinking has several advantages such as low toxicity and absence of crosslinking agents, it also has limitations. One of the most significant issues is the mechanical strength and deficiencies in other mechanical properties. On the other hand, chemically crosslinked hydrogels are relatively more stable and mechanically stronger. In chemical crosslinking methods, two polymer chains are crosslinked through chemical modifications in the presence of multifunctional crosslinkers. Photopolymerization, in particular, offers unique features and advantages compared to other conventional methods [41].

1.3.1 Photopolymerization

In photopolymerization strategy, UV (10-400nm) or visible light (380-700 nm) can be used to initiate polymerization in either linear form or crosslinked form. Spatiotemporal formation of network chains can be regulated such as matrix stiffness, softness, and crosslinking density by controlling the light density, exposure time [42].

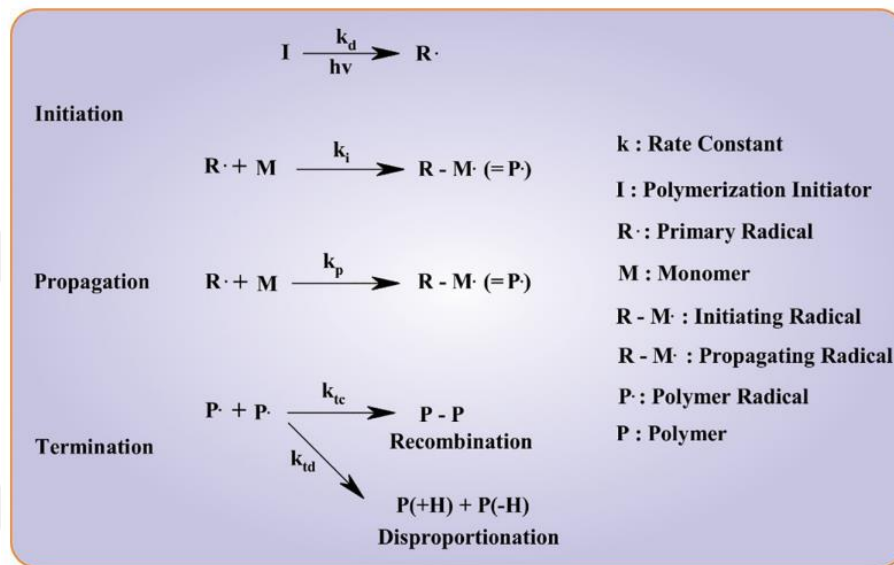


Figure 1.6: Scheme of photopolymerization [42].

Photopolymerization takes place in three steps (Figure 1.6). In the initiation step, photoinitiator is excited by the photons and turn into primary radicals. Primary radicals attack the pi bond in alkene groups in monomers forming a covalent bond one of carbon atoms and make other carbon atom reactive respectively. During the propagation step, monomer units are covalent linked and polymer chains are generated. At the termination stage, chain growth stops when two radical chains combine or participate disproportional reactions which involves pulling hydrogen atom from another reactive chain [42].

There are several advantages of photopolymerization, such as controlling the exposure time, being non-invasive, manipulating the mechanical properties (flexibility, hardness or softness) [43]. Photopolymerization is generally divided into 2 subtypes: UV-induced photopolymerization and Visible Light-induced photopolymerization.

1.3.1.1 UV-induced photopolymerization

UV-induced photopolymerization frequently occurs between 200-400 nm scale. This method allows formation of polymer networks in low primary radicals under mild conditions (room temperature, physiological, aqueous). Although it is an efficient method to form polymer networks, low penetration depth, cytotoxicity, and poor solubility of photoinitiators can be the main drawbacks of UV-induced photopolymerization.

However, it is still widely used for biomaterials studies. In one study a polymeric network using PEG-PEGDA was designed and photodegradation are provided by UV light. Change in UV irradiation time cause hydrogel to undergo changing between sol-to gel or gel to sol[44].

1.3.1.2 Visible Light-induced photopolymerization

Photopolymerization based on visible light is taken place between 380-700 nm scale. This method was proposed in 1970s and used in dental composites [39]. This method has developed by the invention of LED sources. LED provides cleaner and efficient polymerization. In this method, photoinitiator systems are divided into 3 main groups: free radical initiating, ionic initiating and hybrid initiating. For visible light irradiation, type 1 photoinitiators are mostly used [45]. In literature, C.S Bahney and coworkers developed polymeric network that contain mesenchymal stem cells. They used poly(ethylene glycol) diacrylate for hydrophilic characteristic to hydrogel so that bioimmobilization can occur. They defined photo-encapsulation of hMSCs in use of eosin Y, triethanolamine and 1-vinyl-2-pyrrolidinone as photoinitiators [46].

1.4. Postpolymerization of Hydrogels and Reactive Hydrogels

Postpolymerization modification is modification of polymeric materials after synthesis especially with biological molecules. For this purpose, polymers with reactive groups on their chain ends and/or terminal units can be conjugated with DNA, RNA, or protein molecules (Figure 1.7) [47].

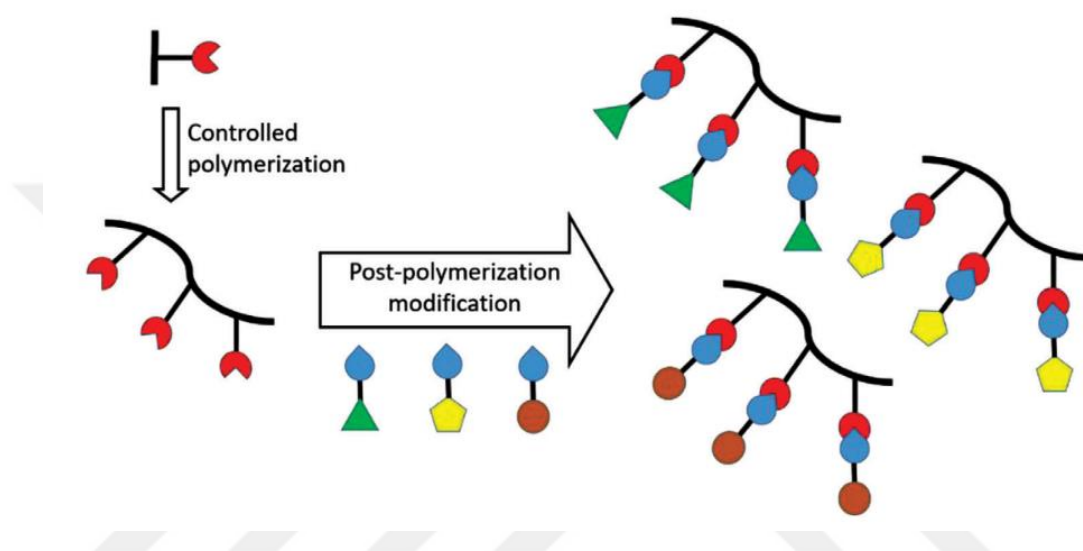


Figure 1.7: Illustration of post-polymerization modification [48].

In order to execute post-polymerization modifications for biotechnologic applications, ‘click reactions’ are appropriate synthesis methods. ‘Click reaction’ term was gained to literature by Sharpless in 2001 [49]. Click reactions are usually preferred in creating reactive functional groups and make polymeric networks functionalized due to some unique advantages; accessible reagents, mild reaction conditions, insensitive to water and air, no side reactions and byproducts. According to Sharpless and his coworkers, click reactions are divided into 4 groups; nucleophilic substitution chemistry, non-aldol carbonyl chemistry, additions to carbon-carbon multiple bonds and cycloaddition chemistry [50]. Schiff’s base reactions are another click chemistry methods that are suitable and useful reactions for bioimmobilization applications by providing reaction between amine and aldehyde groups (Figure 1.9) [51].

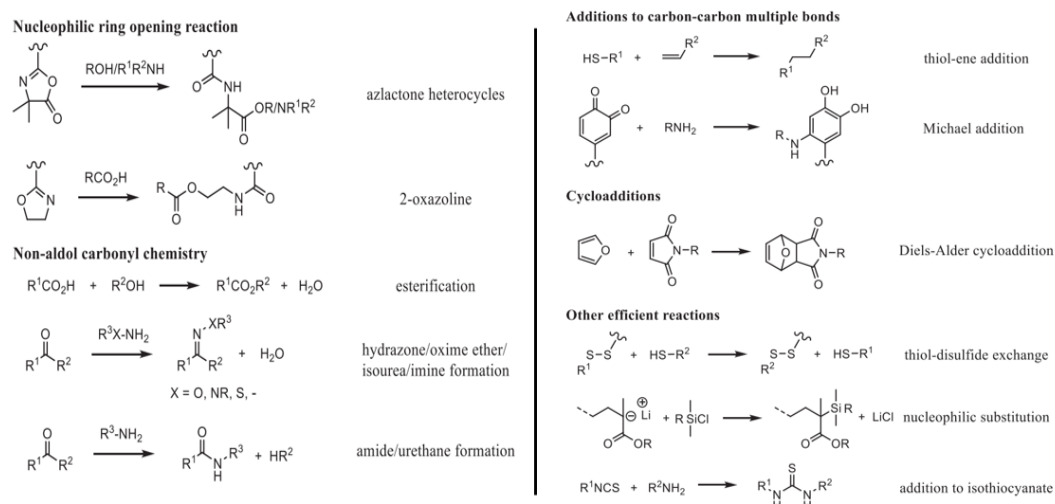


Figure 1.8: Typical click reactions used in polymer modifications. [50].

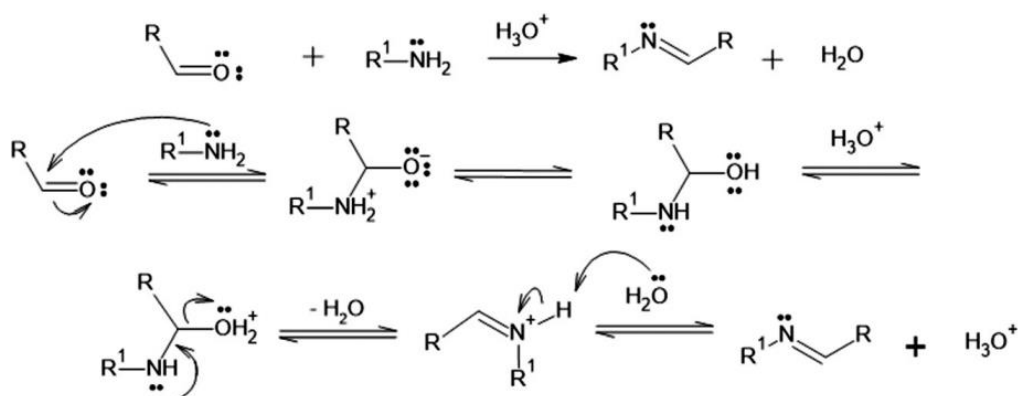


Figure 1.9: Mechanism of Schiff's base reaction between aldehyde and amine groups [51].

The Schiff base reaction generates pH-sensitive dependent reversible covalent bonds between amino and carbonyl groups (ketones and aldehydes). This reaction takes place between pH 4-7 and remains stable at pH 7 and above. However, the carbon-nitrogen double bond tends to dissociate under pH 5 and below. Schiff base reactions are particularly useful for drug delivery since the pH around tumor tissues is around 5 [52]. In one study, Su, Zhang and coworkers obtained aldehyded dextran by oxidation of dextran by using $NaIO_4$. Dextran was then conjugated with doxorubicin via Schiff base reaction, and subsequently cross-linked using ethylenediamine to form nanogels. Since the Schiff base reaction is pH-sensitive, successful drug release from dextran nanogels was achieved (Figure 1.10) [53].

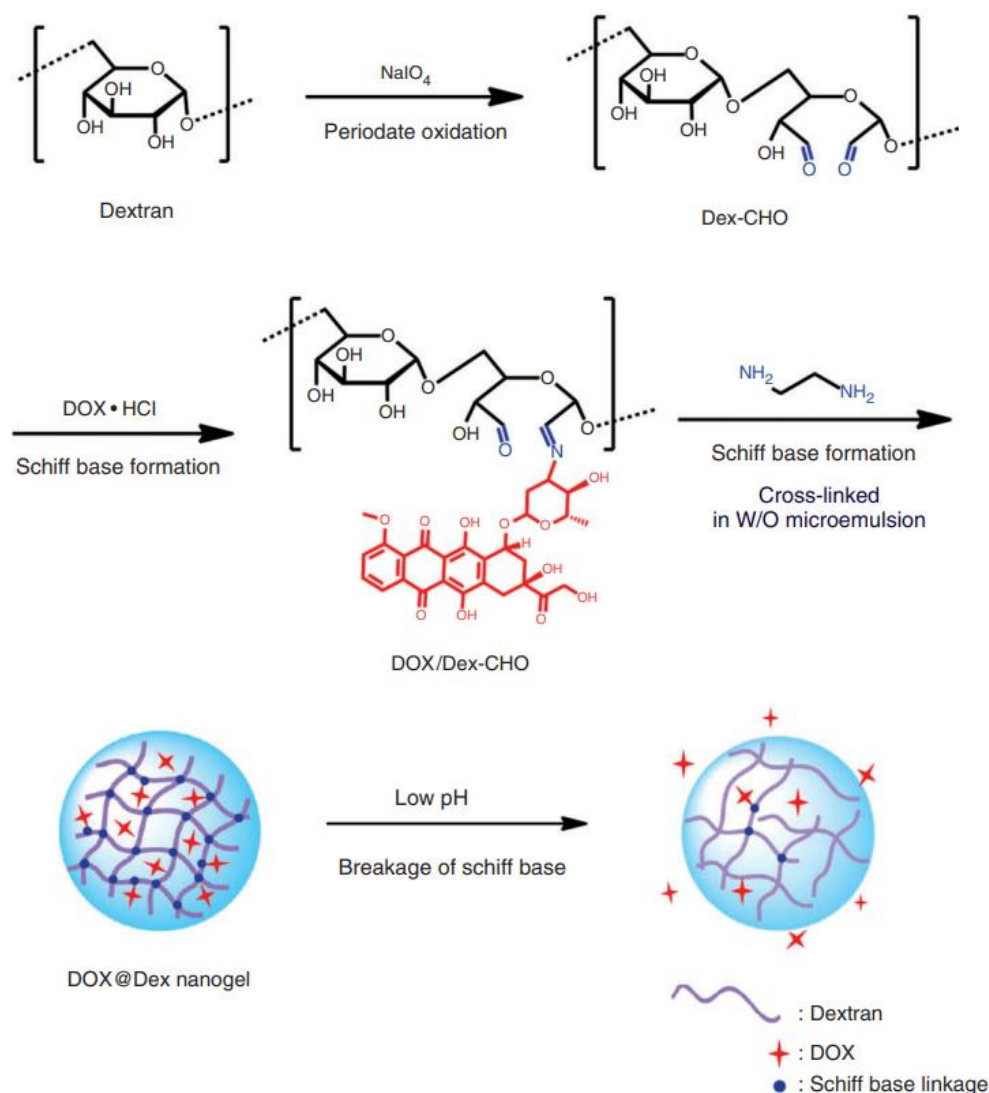


Figure 1.10: Illustration of dextran based nanogels and drug conjugation via Schiff base reaction and drug release in low pH [53].

1.5. Photoresponsive Hydrogels

Hydrogels can be designed to be responsive to environmental factors. Among these methods, light-responsive hydrogels offer several advantages, allowing for control over spatial and temporal features, and facilitating reactions under mild conditions. Therefore, the presence of photolabile or photosensitive groups in polymeric networks is a prerequisite for initiating light-induced photoreactions[54].

Different types of photosensitive materials can be prepared according to photoisomerization, photodimerization, or photocleavage [55]. In photoisomerization polymeric networks contain specific groups such as azobenzene and spiropyran.

These groups can undergo isomerization under UV or visible light irradiation. For instance, azobenzene groups have reversible cis or trans isomerizations due to N=N bonds, resulting in alterations of molecular polarity, molecular size, and even electrical changes [54].

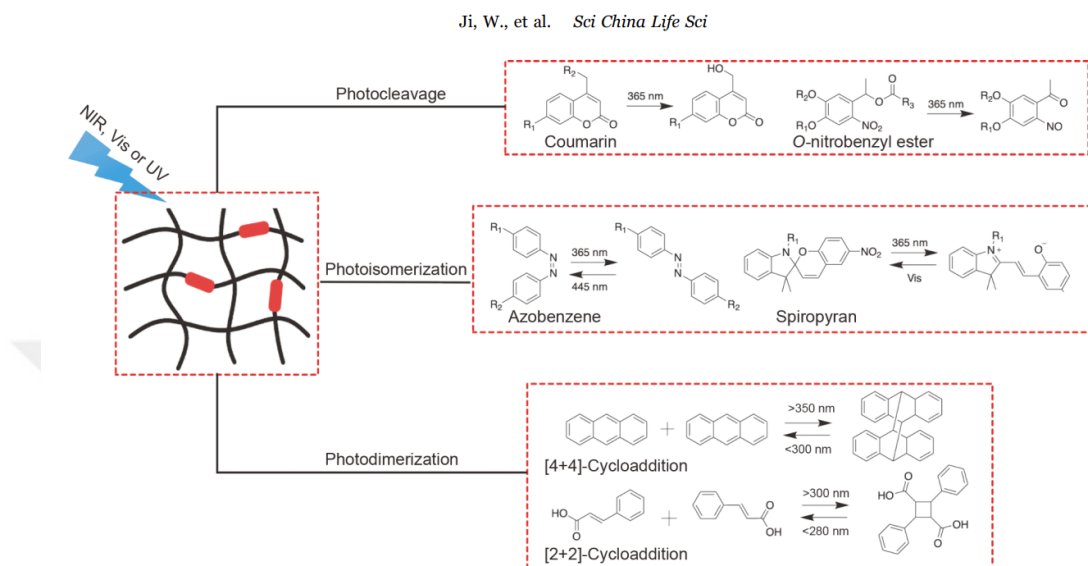


Figure 1.11: Different representative photoreaction types and photosensitive groups for the design of photosensitive hydrogels [55].

Photodimeric hydrogels contain photodimeric groups that undergo reversible crosslinking or de-crosslinking under UV or different light conditions. These transitions initiate gelation or gel-sol transitions, and such physical changes can alter the swelling degree of hydrogels. Basic examples of these photodimeric groups used in hydrogels include coumarin, cinnamic acid, and anthracene[55].

On the other hand, photocleavable hydrogels contain cleavable groups that can be triggered under different wavelength. These hydrogels are used for various purposes, such as cell attachment and release, biomolecule loading, immobilization, and release. The most used in photocleavable hydrogels are o-nitrobenzyl and its derivatives [55]. Using photocleavable groups hydrogel micropatterns can be fabricated. In a particular study, Doh and co-workers designed a terpolymer poly(o-nitrobenzyl methacrylate-co-methyl methacrylate-co-(ethylene glycol) methacrylate) (P(o-NBMA-co-MMA-co-EGMA)) and they produced thin films using this polymer. Prepared thin films were dissolved in patterned manner after UV irradiation. Lastly,

they demonstrated conjugation of biotin to hydroxyl ends of PEG groups allows selective immobilization of streptavidin on surface (Figure 1.12) [56].

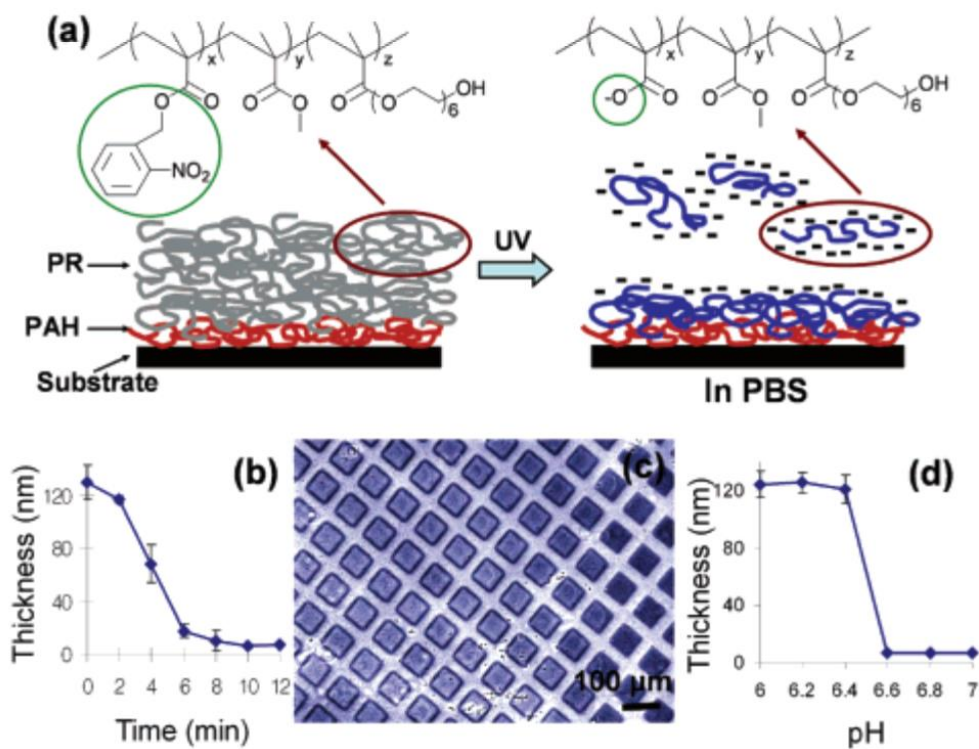


Figure 1.12: Illustration of selective immobilization of biotin and streptavidin on the patterns [56].

1.6. Microwells

Cellular mechanisms are not only governed by genetic materials or intrinsic pathways but also by environmental stimuli. For instance, during the early stages of embryonic cell development in the inner cell mass, cells interact and communicate with each other. This communication occurs through hormones such as paracrine and autocrine, as well as through direct cell-cell interactions or cell-extracellular matrix interaction [57]. Furthermore, mechanical forces imparted from the surrounding environment can potentially alter cellular signals. As mentioned earlier, hydrogels possess the distinct capability to replicate the 3D extracellular matrix. In alignment with this, hydrogel-based microwells also present the same potential. Hydrogel based microwells provide suitable backgrounds for investigating cellular behaviors, measuring low amounts of biomolecules (DNA, RNA, enzymes, polypeptides), and conducting single-cell investigations.

Microfabrication, microwell structures, or micro-scaled topographic patterns can be achieved using lithographic techniques. Specifically, photolithography is one of these techniques, where the pattern of a photomask is transferred onto gel surfaces during exposure to electromagnetic radiation (UV). In photolithography, two distinct approaches can be employed on polymers: either the exposed area could transition from a solid state to a liquid/soluble state due to polymer backbone degradation or the breakage of photolabile crosslinkers, or vice versa. The main advantage of photolithography is the ability to control the pattern shape or create a wide variety of patterns on the surface by regulating exposure time and selecting appropriate monomers and photomasks [58].

On the other hand, soft lithography offers several advantages, including cost-effectiveness and does not require photo-reactive surfaces. This method is primarily based on printing and molding. In soft lithography, an elastomeric stamp, commonly made of poly(dimethylsiloxane) (PDMS), is used to transfer the original pattern onto a third surface. PDMS is a widely used polymeric elastomeric stamp in the literature [59]. For example, Broderick and colleagues, was designed amine-reactive polymer layers on topographically patterned microwells. They utilized polyurethane, which was functionalized through a reactive layer-by-layer assembly of polyethyleneimine and poly(2-vinyl-4,4'-dimethylazlactone). PDMS was used to create the microwells.

Following this, the top surfaces could be coated/immobilized with amine-contained molecules. Since the microwells were also amine-reactive, they could interact with different amine-contained molecules. Consequently, in one scaffold, two different types of amine-contained molecules could be successfully immobilized [57]. In one study, Moeller and colleagues designed a microarray system which was based on microwell structures on hydrogel surface. For this purpose, they used PEG-DA and PEG-MA as crosslinker and monomer respectively. Polymerization was taken place under UV irradiation and microwells were generated through PDMS stamps. After the gelation, they peeled off the stamps and washed surface. They seeded embryonic stem cells (ESs) to obtain Embryonic Bodies (EBs). They showed that they successfully created Embryonic Bodies (Figure 1.13).

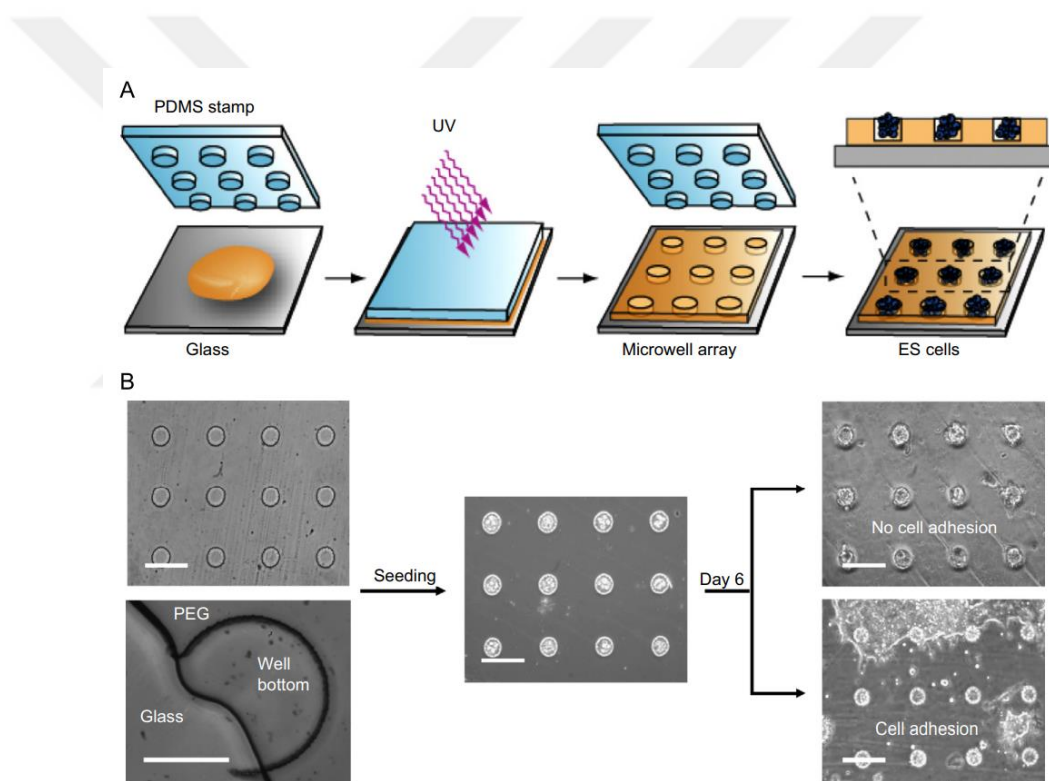


Figure 1.13: Creation of microwell to be used microwell-based microarray systems. [60].

2. AIM OF THE STUDY

The main aim of this study is to prepare photosensitive hydrogels by blue light induced photopolymerization and exposing synthesized hydrogels to UV light (365 nm) through photomask to create amine-reactive microwells for bioimmobilization based applications.

For this purpose, ortho-nitro benzyl ester (ONB) containing hydrogels were synthesized from terephthalic acid according to literature. Three different light responsive hydrogels were synthesized, each incorporating different photosensitive monomers. Also, one hydrogel devoid of ONB unit allowed for control experiments, to confirm that biomolecule immobilizations occurred through Schiff's base reactions.

ONB-contained hydrogels were firstly synthesized under visible blue light and then exposed to UV light (365nm). Therefore, it was demonstrated that, by using ONB groups, polymerization was undertaken successfully, and thus allow subsequent creation of amine-reactive nitrosobenzaldehyde-containing microwells upon UV-irradiation through a photomask.

Prepared microwells could be used for the immobilization of amine-contained small molecules or biomolecules for biosensor application. Also, in future studies by attaching cell binding ligands in the etched region of hydrogels, potential of using micro-structured hydrogels in cell culture based bioapplicatios can be investigated.

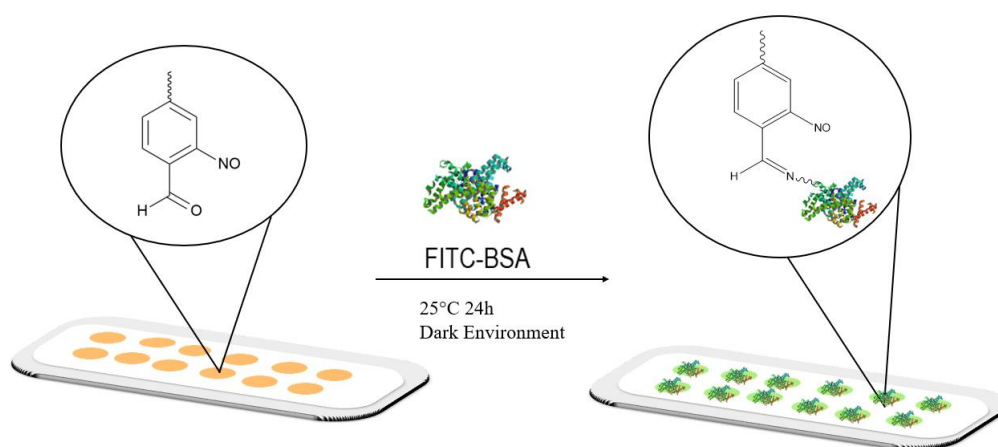


Figure 2.1: General scheme of bioimmobilization of FITC-BSA inside of microwells on hydrogel films.

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Chemicals and Instruments Used in the Thesis

Terephthalic acid (Merck), Sulphuric Acid (H_2SO_4) (Sigma Aldrich), Nitric Acid (HNO_3) (Sigma Aldrich), dH_2O , Ethanol, Sodium Sulfate (Na_2SO_4) (Merck), Borane-Tetrahydrofuran (THF-BH_3) (Sigma Aldrich), ethyl acetate ($\text{C}_4\text{H}_8\text{O}_2$) (EMBOY), triethylamine ($\text{N}(\text{CH}_2\text{CH}_3)_3$) (Merck), acryloyl chloride ($\text{C}_3\text{H}_3\text{ClO}$) (Sigma Aldrich), dichloromethane (CH_2Cl_2) (ISOLAB Chemicals), hexane (C_6H_{14}) (EMBOY), Silica, dimethyl sulfoxide ($\text{C}_2\text{H}_6\text{OS}$) (Merck), ; Poly(ethylene glycol) methyl ether methacrylate (PEGMEMA) Mw: 500 (Merck), Di(ethylene glycol) methyl ether methacrylate (DEGMEMA) Mw:188.22 (Merck), Phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide (Sigma Aldrich). Sodium Hydroxide (NaOH) (ISOLAB Chemicals), 1x1.5 cm lamels, cover glasses, acetone ($\text{C}_3\text{H}_6\text{O}$) (ISOLAB Chemicals), Toluene (C_7H_8) (Merck), hexadecyltrimethoxysilane, methanol (CH_3OH) (ISOLAB Chemicals), trimethoxysilyl propyl methacrylate (TMSMA) (Sigma Aldrich) Fluoresceinamine, Isomer I Mw: 347.32 (Merck), 4-(trifluoromethyl)benzylamine (TFBA), Bovine Serum Albumin (BSA) Sigma Aldrich. Hand-made Blue Light Source: Perium Model S-50-12 250V 50/60 Hz. (Consisting of 65 LED light) positioned on one of the flat surfaces of a wooden box measuring (16.5x16.5x20 cm

3.2. Devices

Table 3.1: Instruments used in Thesis Study.

Name	Model	Location
Rotary Evaporator	Heidolph	Gebze Tecnical University
FTIR Spectrophotometer	Perkin Elmer 100	Gebze Tecnical University
NMR Spectrophotometer	Varian INOVA 500 MHz	Gebze Tecnical University
Scanning Electron Microscope (SEM)	Thermo Fisher Scientific	Gebze Tecnical University
UV Light Source	Blak-Ray UV Lamp (365nm, 100 watt)	Gebze Tecnical University
Fluorescent Microscope	Zeiss Observer Z1	Boğaziçi University

3.3. Synthesis of Light Responsive Crosslinker

3.3.1. Synthesis of Nitroterephthalic Acid

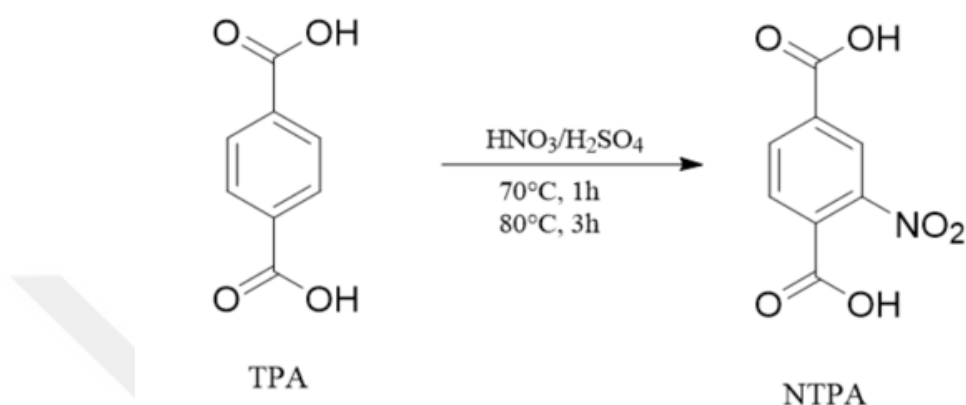


Figure 3.1: Synthesis of NTPA.

Terephthalic acid (200 g) was taken in a 250 mL round-bottom flask (RBF) with a stir bar. The RBF was placed in an ice-filled container and cooled to 0°C . H_2SO_4 (250 mL) was slowly added. The RBF was then transferred to an oil bath and heated to 70°C . HNO_3 (16 mL) were added gradually. After one hour, the temperature was increased to 80°C and left for three hours.

After the mixture was cooled to room temperature, it was poured into an ice-filled beaker. The solution was filtered multiple times with distilled water until the yellowish color disappeared. Subsequently, the solution was filtered with 500 mL of distilled water at 70°C , followed by 200 mL of ethanol at 70°C . The leached solution was dried using Na_2SO_4 , and any solid Na_2SO_4 was removed from the solution. The ethanol was evaporated under vacuum. $^1\text{H-NMR}$ was used for characterization.

3.3.2. Synthesis of (2-Nitro-1,4- phenylene dimethanol) NPDM

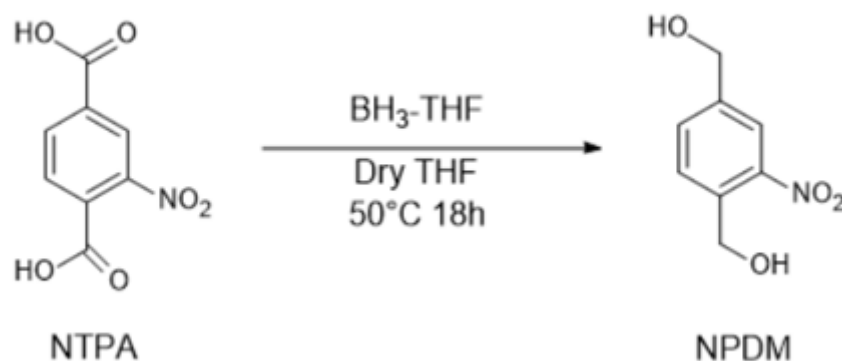


Figure 3.2: Synthesis of NPDM crosslinker.

Synthesis was carried out according to literature procedure [61]. NTPA (1.175 g) was weighed and taken into 100 mL RBF with a stirbar then dried under vacuum for 2 hours. NTPA was dissolved in anhydrous THF under Ar. The RBF was placed in an ice-filled container, and 25 mL of THF-borane solution was slowly added. Then the RBF was removed from the ice and left at room temperature for 1 hour. Subsequently, it was transferred to a 50°C oil bath and the reaction was allowed to proceed for 21 hours.

5 mL dH₂O was added into RBF and waited until the colour of solution turned to yellow. THF and water was vaporized by rotary evaporator at 36°C, 200 mbar. 200 mL ethyl acetate and 20 mL dH₂O was added and extraction was taken place at room temperature. Na₂SO₄ was added for drying and and solution was vaporized. Structure was confirmed by using H-NMR analysis.

3.3.3. Synthesis of (2-Nitro-1,4- phenylene bismethylene diacrylate (NPDMA)

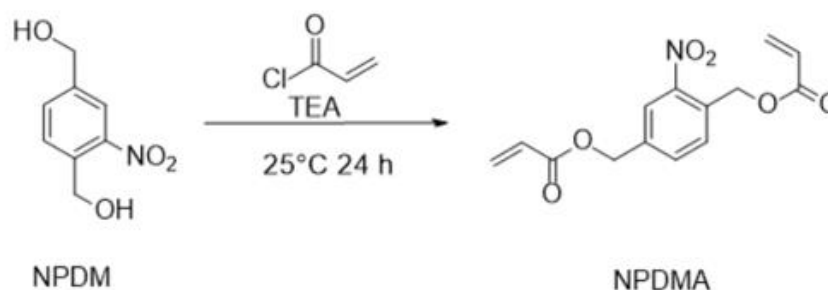


Figure 3.3: Synthesis of NPDMA crosslinker.

Synthesis was carried out according to literature procedure. [61] NPDM (0.93 mg) was weighed and taken into 100 mL RBF with a stir bar. 26 mL anhydrous THF was added to the mixture and stirred, 3.7 mL triethylamine was added subsequently. Solution was taken into ice-filled container and cooled down to 0°C. Acryloyl chloride (1.3 mL) was slowly added into solution and mixture was heated up to 25°C and stirred for 24 h.

Solvent was vaporized and substance was dried under vacuum. Obtained product was purified by using column chromatography (ethyl acetate:hexane) was used for purification. ¹H-NMR spectroscopy was utilized to confirm the structure.

3.4. Glass Surface Preparation

3.4.1. Fabrication of Acrylated Glass

NaOH (5 g) was weighted and added into 500 mL- Erlenmeyer Flask and dissolved in 200 mL dH₂O. 30 pieces of glass (1.25x1.25 cm) were taken into solution and left there for 24 hours. The glasses were then rinsed with dH₂O three times and subsequently washed with acetone three times. Finally, they were dried at 70°C for 1.5 hours.

50 mL of anhydrous toluene was poured into an Erlenmeyer flask, followed by the addition of 0.5 mL of 3-(trimethoxysilyl)propyl methacrylate (TMSMA). Clean and dry glass pieces were added to the solution one by one. The solution was then homogenized using a sonicator for 15 minutes and left for 24 hours. The glasses were subsequently washed three times with 50 mL of toluene and 50 mL of methanol. Afterward, they were dried using a stream of Ar flow, placed into petri dishes, and stored at +4°C.

3.4.2. Fabrication of Hydrophobic Cover-Glass

NaOH (5 g) was weighted and added into 500 mL- Erlenmeyer Flask and dissolved in 200 mL dH₂O. 30 pieces of cover-slips were taken into solution and left there for 24 hours. The glasses were then rinsed with dH₂O three times and subsequently washed with acetone three times. Finally, they were dried at 70°C for 1.5 hours.

20 mL of anhydrous toluene was poured into an Erlenmeyer flask, followed by the addition of 0.4 mL hexadecyltrimethoxysilane. Clean and dry glass pieces were added to the solution one by one. The solution was then homogenized using a sonicator for 15 minutes and left for 24 hours. The glasses were subsequently washed three times with 50 mL of toluene and 50 mL of methanol. Afterward, they were dried using a stream of Ar flow, placed into petri dishes, and stored at +4°C.

3.5. Preparation of Hydrogel Precursor Solution

For the synthesis of S-0 hydrogel, 2 mL eppendorf tube was taken and 71.4 μ L PEGMEMA, 20.5 μ L PEGDA, 50 μ L photoinitiator (BAPO) and 50 μ L THF were added into tube. Precursor solution was gently stirred by thin glass stick.

For the synthesis of S-5 hydrogel 2 mL eppendorf tube was taken and 71.4 μ L PEGMEMA, 2.9 mg NPDMA 15.375 μ L PEGDA, 50 μ L photoinitiator (BAPO) and 50 μ L THF were added into tube. Precursor solution was gently stirred by thin glass stick.

For the synthesis of S-10 hydrogel 2 mL eppendorf tube was taken and 71.4 μ L PEGMEMA, 5.8 mg NPDMA 10.25 μ L PEGDA, 50 μ L photoinitiator (BAPO) and 50 μ L THF were added into tube. Precursor solution was gently stirred by thin glass stick.

For the synthesis of S-20 hydrogel 2 mL eppendorf tube was taken and 71.4 μ L PEGMEMA, 11.6 mg NPDMA, 50 μ L photoinitiator (BAPO) and 50 μ L THF were added into tube. Precursor solution was gently stirred by thin glass stick.

3.6. Synthesis of Hydrogels on Glass Surfaces

For each hydrogel precursor solution, eight pieces of acrylated glass were taken. 20 μ L of the precursor solution was dropped onto each glass. The acrylated glasses were covered with hydrophobic cover glasses. The glasses were exposed to a visible-blue light source for 30 minutes. After removing the hydrophobic cover glasses, the surfaces were washed with THF three times and dried under airflow. The dried glasses were then placed in a vacuum oven at room temperature for 18 hours. The presence of ONB units in the gel structure was confirmed via FTIR spectroscopy.

3.7. Activation of Hydrogels via Photodegradation By UV

3.7.1. Amine Reactive Hydrogel Formation

The hydrogel-coated glasses were positioned under a UV source with a distance of 6.4 cm between the source and the glass. The hydrogels were exposed to the UV source for 30 minutes. They were then washed with THF three times and subsequently placed in an oven at 25°C for 1.5 hours. The hydrogels on glass surfaces were then analyzed using FTIR spectroscopy.

3.7.2. Formation of Micropattern/Microwell in Hydrogel Coated Glasses

Photomasks were placed onto hydrogel-coated glasses. Hydrogels were exposed to UV light through photomask for 30 minutes. Afterward, they were washed with THF three times and placed in an oven at 25°C for 1.5 hours. The hydrogels on the glass surfaces were subsequently analyzed using FTIR spectroscopy. The formation of micropatterns was monitored using a light microscope and ESEM.

3.8. Immobilization of TFBA and Biomolecules on Hydrogels

3.8.1. 4-(Trifluoromethyl)benzylamine (TFBA) Immobilization

10 μ L 4-(Trifluoromethyl)benzylamine (TFBA) was dissolved in DMF (400 μ L). 20 μ L solution was carefully spread over the glass UV activated hydrogel films (1.25x1.25 cm) and left for 24 h. Hydrogels were washed with DMF for 1 time and THF for 2 times. They were dried in evacuated oven at 25°C for 1.5 h. This process was applied for all hydrogel coated glasses (S-0, S-5, S-10, S-20). Surfaces were analyzed by FTIR spectroscopy.

3.8.2. Immobilization of Fluoresceinamine on UV-Activated Hydrogel Coated Glasses

2.3 mg Fluoresceinamine was dissolved in DMF (460 μ L). 20 μ L fluoresceinamine solution was spreaded onto 1.25x1.25 cm UV activated smooth or patterned hydrogels. Cover-glasses were placed onto glasses and left for 24 h. Hydrogels were washed with DMF for 1 time and THF for 2 times. They were dried in evacuated oven at 25°C for 1.5 h. This process was applied for all hydrogel coated glasses (S-0, S-5, S-10, S-20). Surfaces were visualized by fluorescence microscopy.

3.8.3. Immobilization of Fluorescent BSA on UV-Activated Hydrogel Coated Glasses

0.26 mg FITC-BSA was dissolved in 260 μ L PBS (pH=7.5). 10 μ L of solution was slowly added onto micro patterned hydrogel films on glass. They were placed into petri dishes and left for 24 h. Surfaces were washed with PBS (pH=7.5) for 3 times and with dH₂O 3 times with. They were dried in oven 25°C for 1.5. Optic results were received by fluorescence microscopy.

4. RESULTS and DISCUSSION

4.1. Synthesis of Nitroterephthalic acid (NTPA)

Nitroterephthalic acid was synthesized starting from terephthalic acid according to literature procedure [61]. After the synthesis, structural characterization was carried out by FTIR and $^1\text{H-NMR}$. Characteristic symmetric stress and asymmetric stress peaks of nitro groups was observed in 1536 cm^{-1} and 1355 cm^{-1} respectively (Figure 4.1.1). $^1\text{H-NMR}$ results demonstrates that presence of three peaks between 7.96-8.44 ppm indicates aromatic protons on the benzene ring which are shown by a, b and c. (Figure 4.1.2). Peak at 8.5 ppm showed dinitro based impurity, which is calculated as 3.5%. FTIR and HNMR data were then compared with the literature study from which the protocol was adopted, confirming the successful synthesis of the compound.

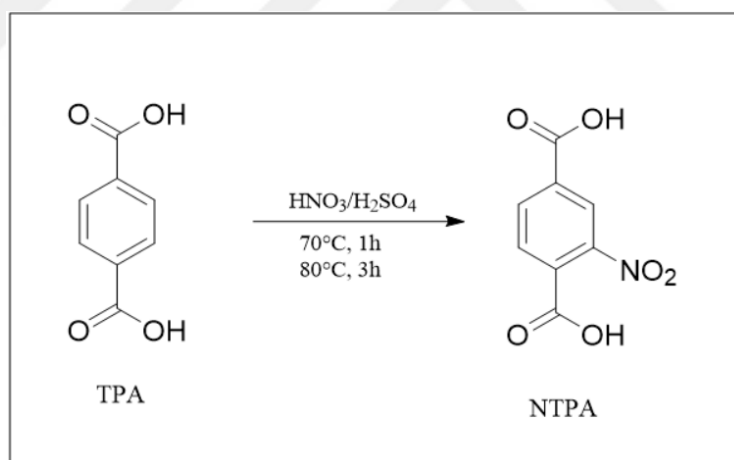


Figure 4.1: Synthesis of NTPA diacid.

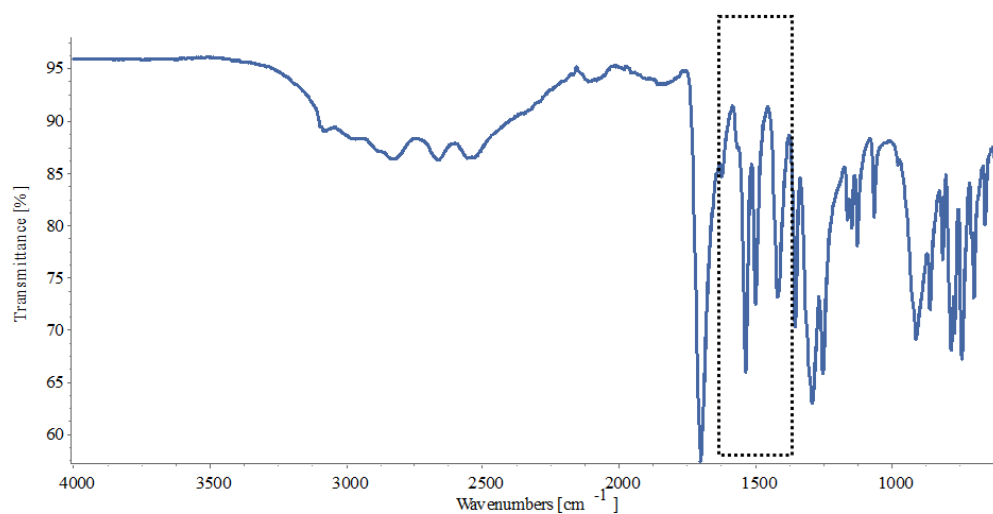


Figure 4.2: FTIR spectrum of NTPA diacid.

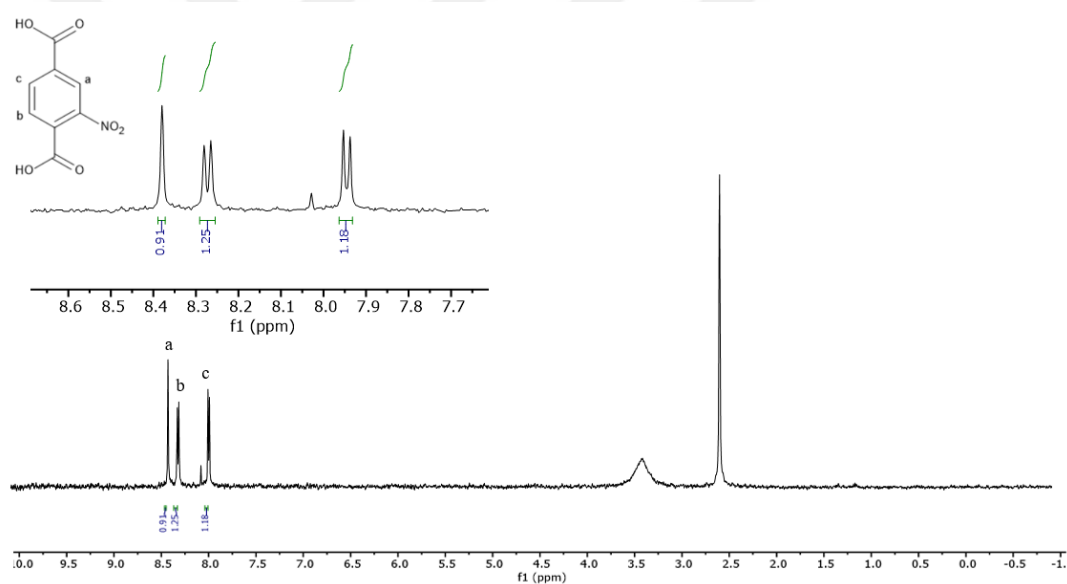


Figure 4.3: ¹H-NMR spectrum of NTPA diacid.

4.2. (2-Nitro-1,4-phenylene) dimethanol (NPDM) Synthesis

The synthesis of (2-Nitro-1,4-phenylene) dimethanol (NPDM) was carried out according to literature protocol. The reaction was taken place in the presence of NTPA and Borane-THF in 50°C for 18h (Figure 4.2.1). FTIR and $^1\text{H-NMR}$ were used for structural characterization. In the FTIR spectra, the disappearance of the peak at 1704 cm^{-1} , which corresponds to the carboxylic acid, and the presence of the -OH stretching peak at 3300 cm^{-1} indicate the successful synthesis of NPDM (Figure 4.2.2). Furthermore, in the $^1\text{H-NMR}$ analysis, in addition to the aromatic protons, the appearance of the CH_2 protons confirms the successful formation of NPDM (Figure 4.2.3). The synthesized substances were subsequently compared with the literature study, revealing their compatibility. A specific peak at 8.05, which was attributed to the presence of dinitro based impurity, notably decreased. This outcome further indicates the purity of the high-resolution ($^1\text{H-NMR}$) result, aligning with the findings in the literature study. [61].

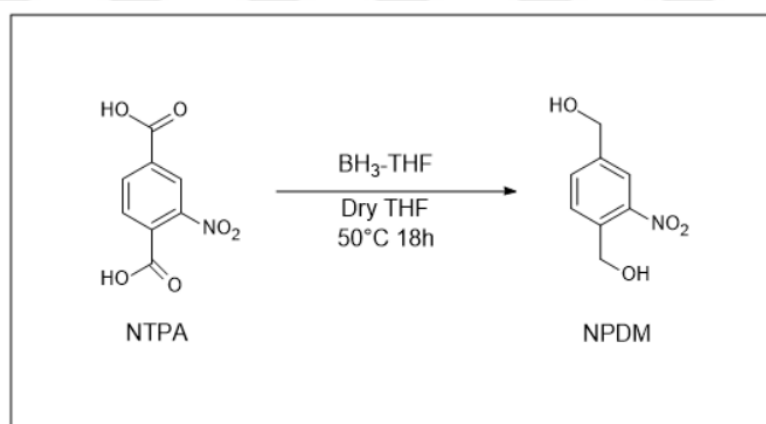


Figure 4.4: Synthesis of NPDM diol.

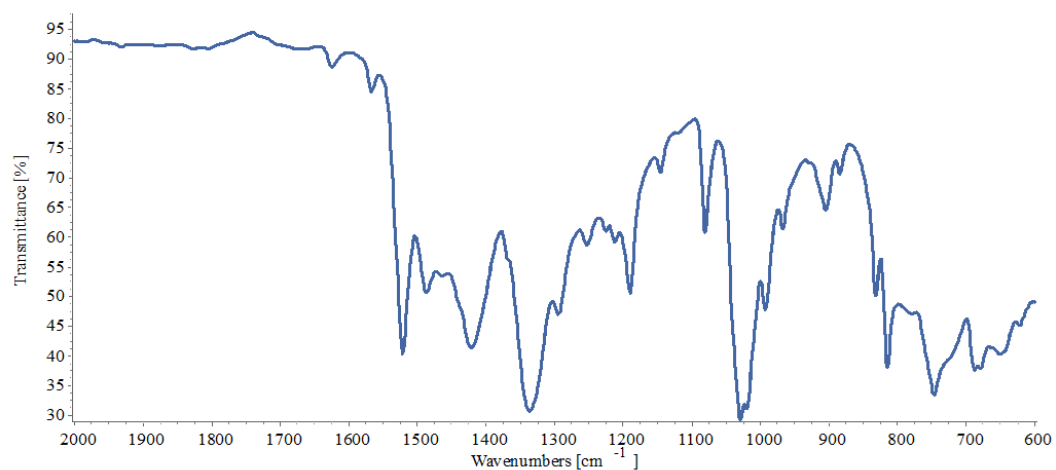


Figure 4.5: FTIR spectrum of NPDM crosslinker.

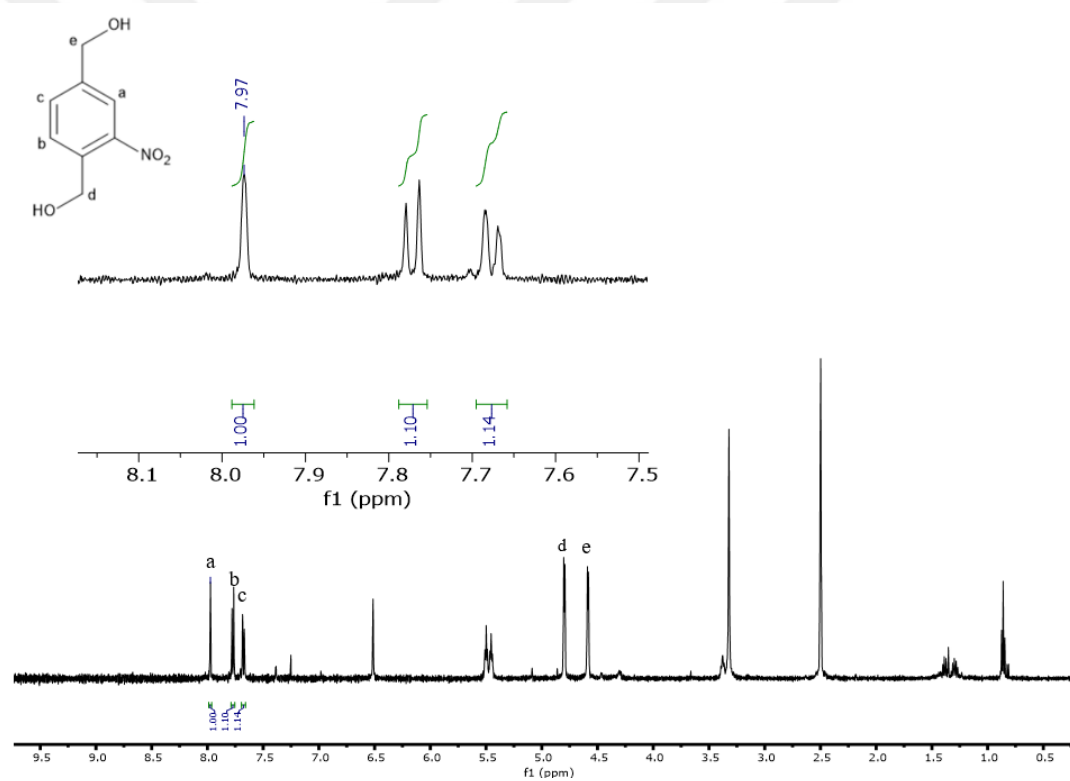


Figure 4.6: ¹H-NMR spectrum NPDM crosslinker.

4.3. Synthesis of (2-Nitro-1,4-phenylene) bis(methylene) diacrylate

The NPDM molecule synthesized in the previous step was reacted with acryloyl chloride in the presence of TEA at room temperature for 24h and purified by column chromatography (Figure 4.3.1). Structural characterization was carried out by FTIR and $^1\text{H-NMR}$. In the FTIR spectrum, 1727 cm^{-1} C=O stretching of ester groups and $1606\text{-}1616$, 1405 and 810 cm^{-1} indicate $\text{CH}_2=\text{CH-R}$ stress, $\text{CH}_2=\text{CH-R}$ shear, $\text{CH}_2=\text{CH-H}$ out-of-plane bending vibrations was observed respectively (Figure 4.3.2). In $^1\text{H-NMR}$, CH_2 protons belonging to acrylate groups were also detected besides the presence of aromatic peaks (Figure 4.3.3). These results show synthesized NPDMA molecule is compatible with the literature report [61].

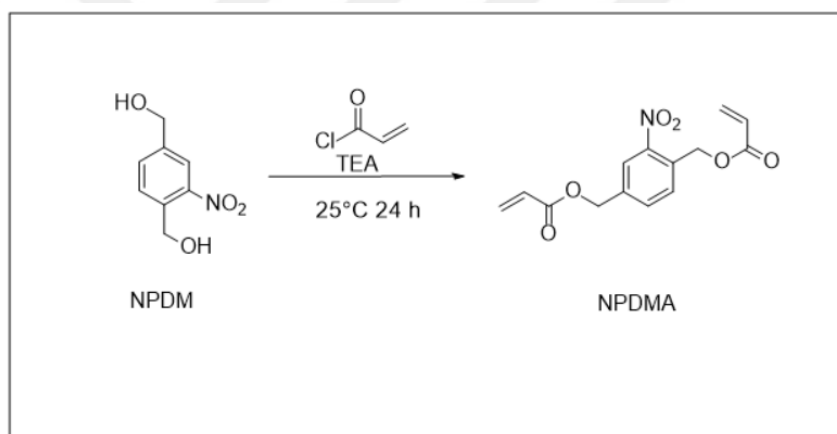


Figure 4.7: Synthesis of NPDMA crosslinker.

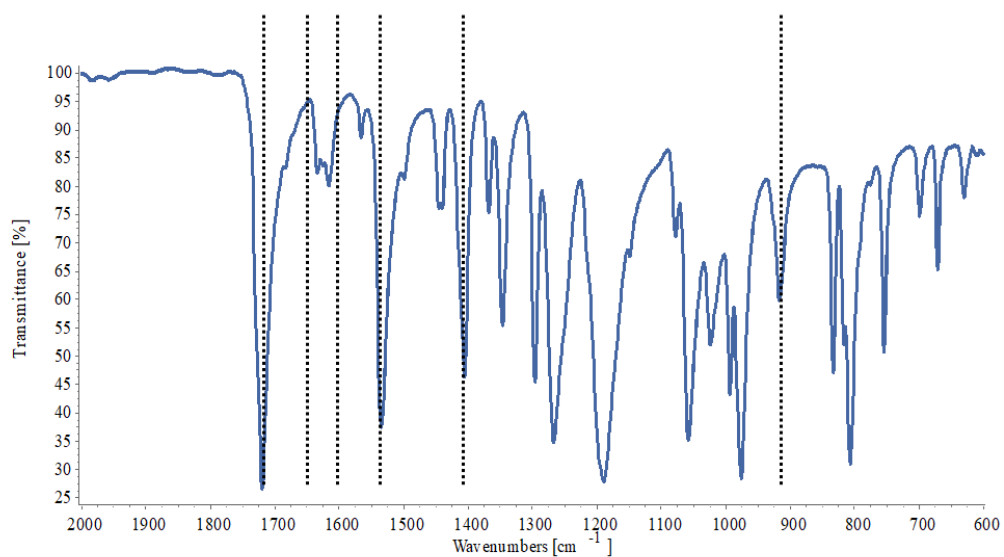


Figure 4.8: FTIR spectrum of NPDMA crosslinker.

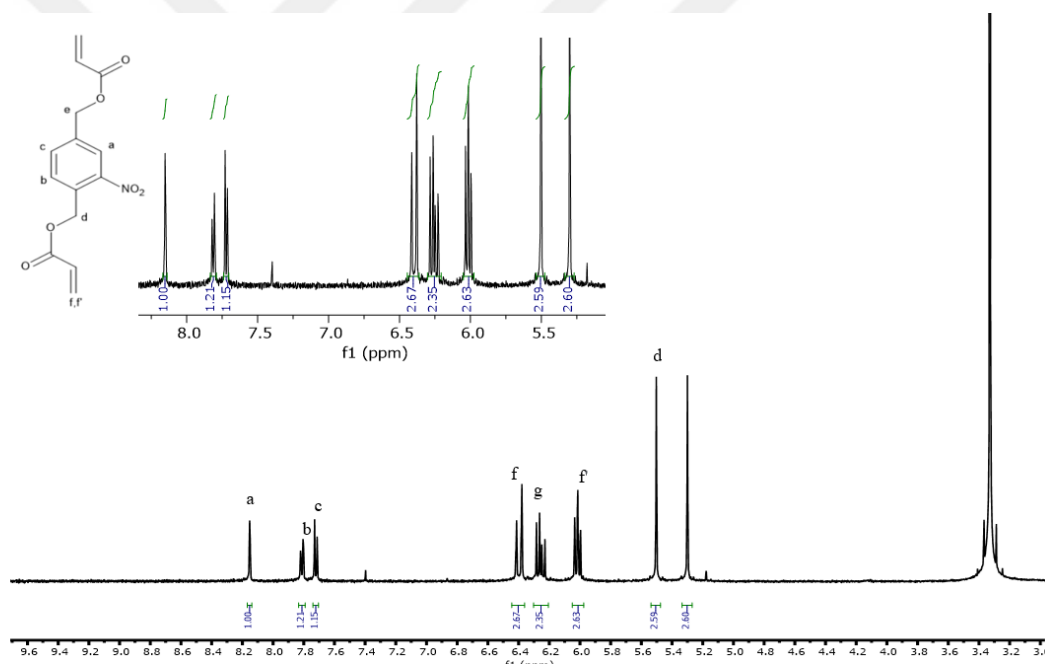


Figure 4.9: ¹H-NMR spectrum of NPDMA crosslinker.

4.4. Synthesis of Hydrogel on Glass Surface via Photopolymerization

After synthesizing the light-responsive crosslinker, three different hydrogel films were prepared by adjusting its molar ratio in the hydrogel precursor to 5%, 10%, and 20%, and they were respectively named S-5, S-10, and S-20. To achieve a total crosslinker ratio of 20%, PEGDA was also added at concentrations of 15% and 10% for S-5 and S-10, respectively. As part of the control experiments, hydrogel films containing only PEGDA as the crosslinker were also synthesized. (S-0). For the synthesis, a 20 μ L solution was taken and dropped onto an acrylated glass surface. The acrylated glasses were covered with hydrophobic cover slides and placed under a blue light source (Figure 4.10). The polymerization time was set to 30 minutes. At the end of the 30-minute period, gels were washed with THF and dried in oven. Obtained hydrogel films were colorless and transparent (Figure 4.11).

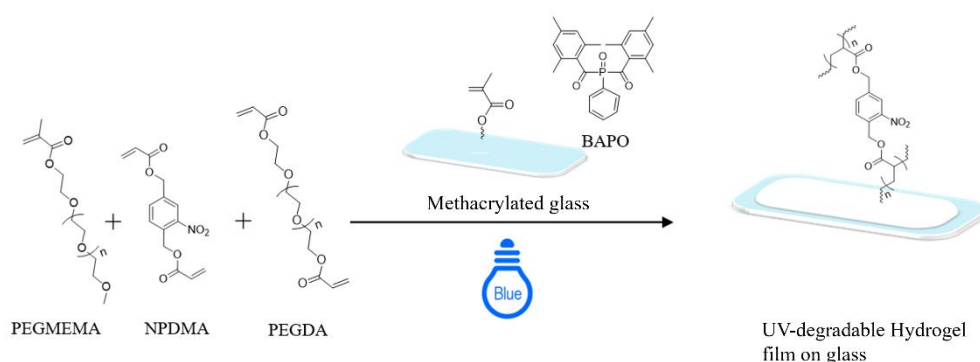


Figure 4.10: Illustration of coating of methacrylated glass with hydrogel film.



Figure 4.11: Photographs of hydrogel coating on glasses.

For structural characterization, hydrogel films were analyzed by FTIR spectroscopy. It was observed that peak at 1536 cm^{-1} gradually increased from S-5 to S-20 (Figure 4.12), while it was absent in S-0 (Figure 4.12). These FTIR results indicate that the concentration of the nitrobenzyl unit in the final hydrogel can be adjusted by varying the ratio of NPDMA in the hydrogel precursor.

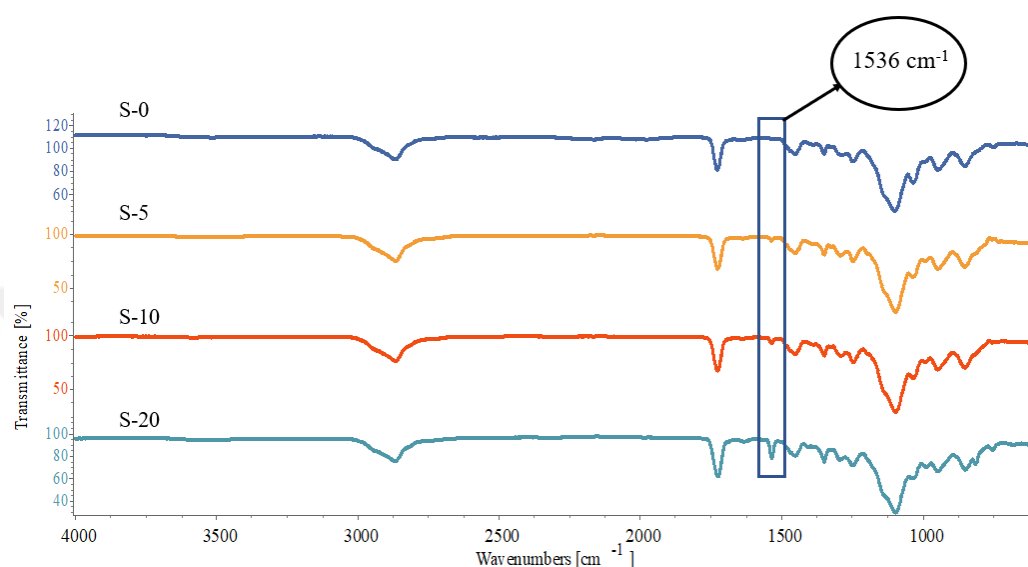


Figure 4.12: FTIR spectra of photopolymerized hydrogels containing NPDMA groups.

4.5. Formation of Nitrosobenzaldehyde Groups by UV Exposure

To generate reactive hydrogels, the fabricated surfaces were exposed to 365 nm UV light for 30 minutes. During this exposure, the nitrobenzaldehyde groups were anticipated to decompose, forming nitrosobenzaldehydes. After the 30-minute period, the glass surfaces changed in color from colorless and transparent to a yellow-orange hue. In contrast, there was no color change observed in the control hydrogel (S-0). As the concentration of NPDMA crosslinker increased in the gel precursor solution, a noticeable shift in color towards orange was observed (Figure 4.13). To validate the structure, presence of nitro group was determined by FTIR analysis. The peaks at 1536 cm^{-1} in S-5, S-10, and S-15 disappeared, indicating the decomposition of the ortho-nitrobenzyl units (Figure: 14). The decomposition of ONB groups into nitroso benzaldehyde groups under UV irradiation is already known in the literature. One can expect to see aldehyde peaks to prove nitroso

benzaldehyde formation; however, in our system, we did not observe these peaks due to the presence of ester units in hydrogels. It was thought that the aldehyde related carbonyl peaks around 1700 cm^{-1} are under ester related carbonyl peak. Nevertheless, the conversion of NO_2 groups to NO groups caused the disappearance of a specific peak at 1536 cm^{-1} . This disappearance provides evidence for the cleavage of ONB ester units into nitrosobenzaldehyde and carboxylic acid. Taken together, we confirm that the generation of nitroso benzaldehyde groups was successfully carried out.



Figure 4.13: Photographs of hydrogel coating on glasses after UV exposure.

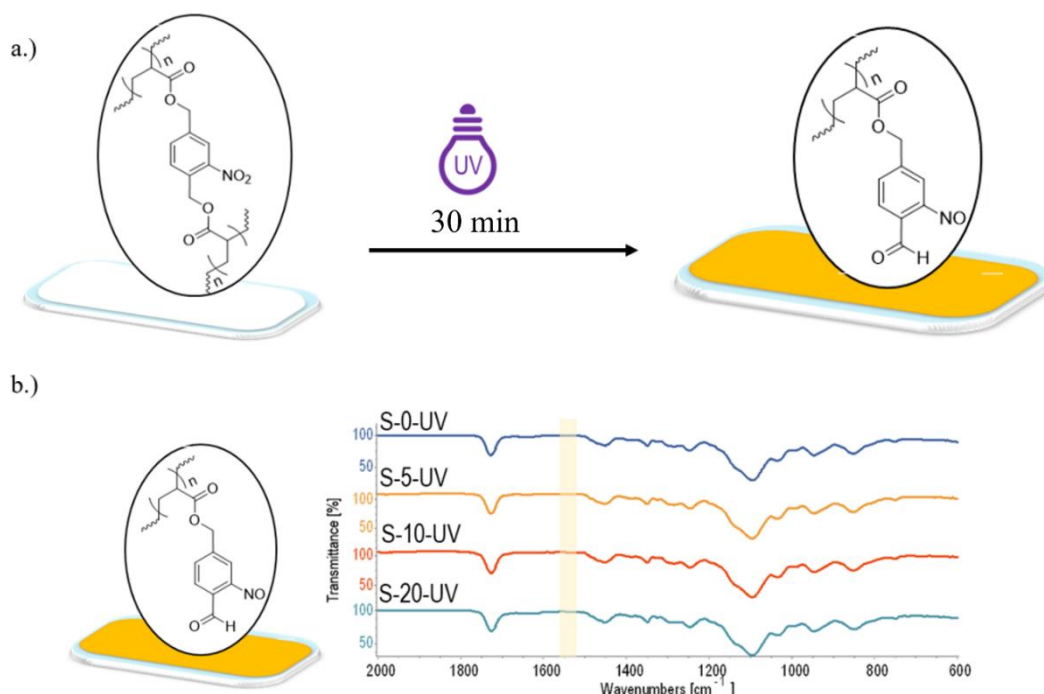


Figure 4.14: a. General scheme of decomposition of nitrobenzyl groups into nitrosobenzaldehyde group under UV b. FTIR spectra of related hydrogels.

4.6. Conjugation of UV-Activated Hydrogels with Amine-Containing Molecules

4.6.1. Conjugation of TFBA onto UV exposed hydrogels

Exposure to UV light is expected to cause the decomposition of hydrogel, resulting in the formation of nitrosobenzaldehyde groups. These aldehyde bearing groups are expected to be reactive towards amine-bearing molecules. An amine-containing molecule, namely TFBA, was used to confirm the amine reactivity of the hydrogel film (Figure 4.15.a). This molecule was selected due to its easily detectable C-F stretching vibration in FTIR analysis. In the FTIR analysis, the appearance of a peak at 1326 cm^{-1} corresponding to the C-F stretching vibration indicated successful conjugation. It was also observed that increasing the ratio of NPDMA in the hydrogel precursor led to an increase in the peak intensity of the C-F bond, suggesting that the density of immobilized groups could be adjusted by changing the amount of ONB ester containing crosslinker (Figure 4.15.b).

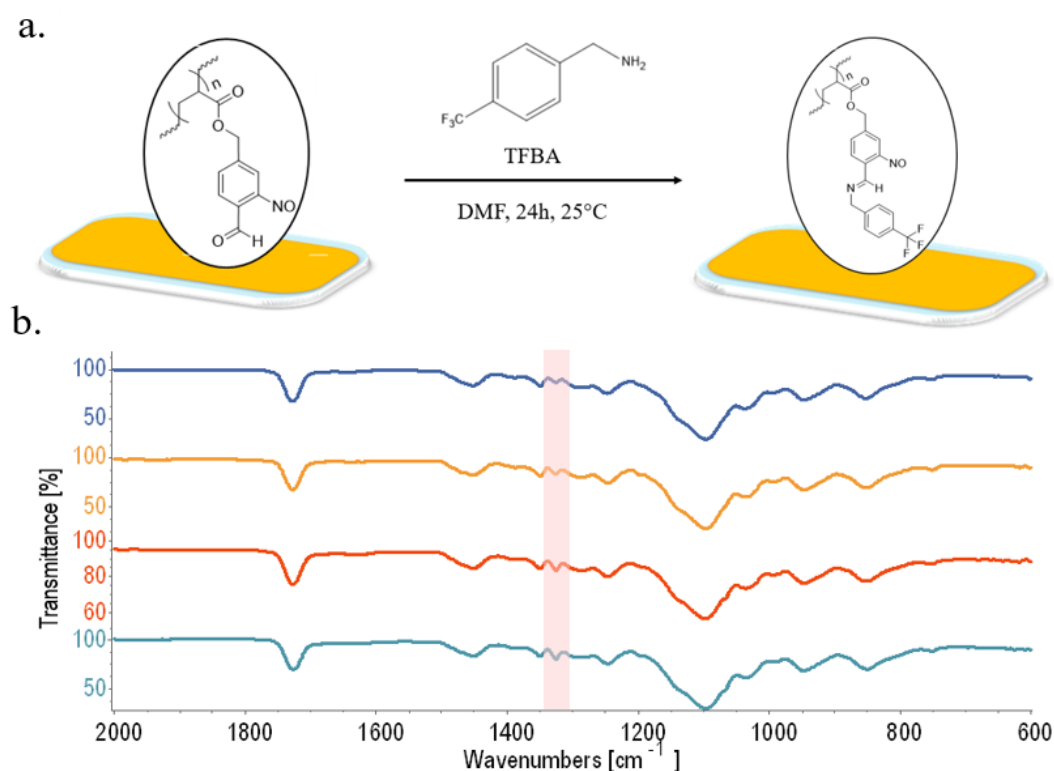


Figure: 4.15: a) General scheme of conjugation of TFBA molecules with reactive surface. b) FTIR spectra of conjugation of TFBA with amine-reactive surface.

4.6.2. Control Experiment of TFBA Immobilization by using Acrylic Acid Containing Hydrogels

So far, we have demonstrated that hydrogels containing ONB ester acquire amine reactivity upon exposure to UV light, attributed to the formation of nitroso benzaldehyde units. However, it's important to note that the degradation of ONB ester can yield carboxylic acid units in addition to nitroso benzaldehyde. Consequently, one might consider the possibility that the resulting carboxylic acid could also contribute to the immobilization of amine groups onto the hydrogel. Even though this scenario does not alter the focus of our research, we felt it was essential to establish a control experiment. To address this, acrylic acid hydrogels were synthesized under blue light using a mixture of PEGMEMA, acrylic acid, and PEGDA in a 60:20:20 ratio (Figure 16). Subsequently, these hydrogels were immersed in a TFBA solution under the same conditions as the previous hydrogels. As depicted in Figure 4.17, the peak related to C-F stretching vibration at 1536 cm^{-1} exhibited only a slight increase. Given that there was already a peak in the untreated hydrogel spectrum within this region, it can be inferred that this increase lacks significance. This spectrum was further compared with the FTIR spectroscopy of TFBA-conjugated S-20 (Figure 4.18). The FTIR spectroscopy results clearly indicate a substantial distinction in the specific C-F asymmetric stretch vibration at 1536 cm^{-1} . Therefore, it can be said that the amine reactivity of the UV-exposed hydrogel primarily stems from the photogenerated nitroso benzaldehyde groups.

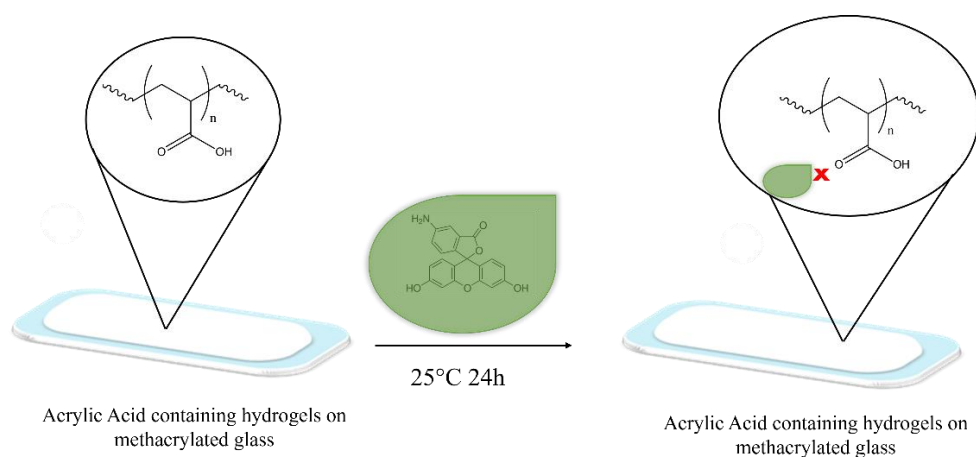


Figure 4.16: Illustration of coating of methacrylated glass with hydrophilic acrylic acid containing hydrogel.

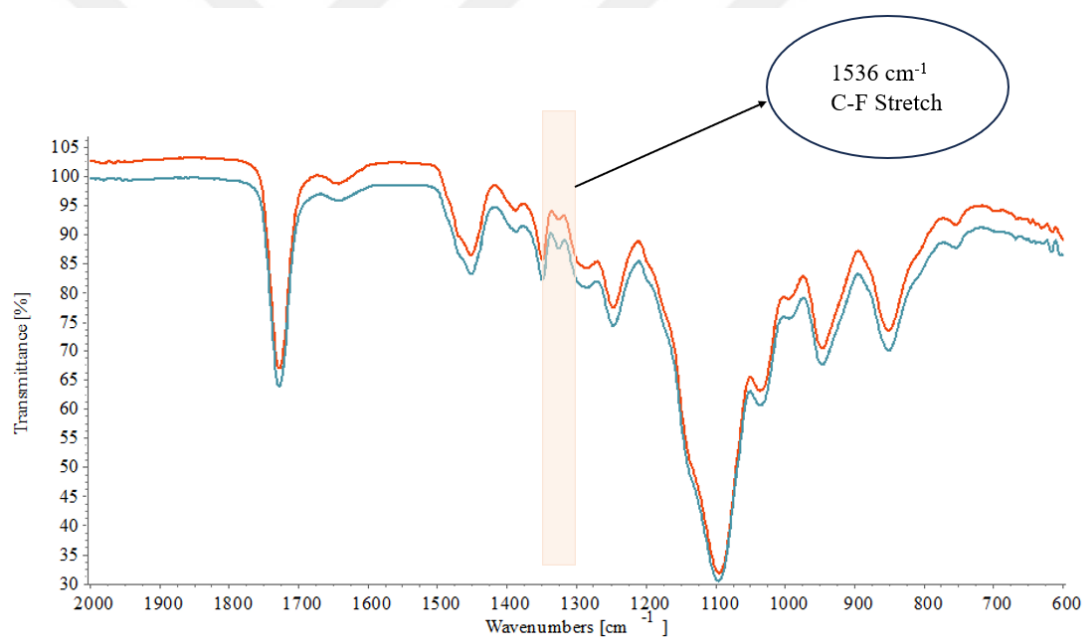


Figure 4.17: FTIR spectra of TFBA-incubated acrylic acid-containing hydrogels (blue) and untreated acrylic acid hydrogels (red).

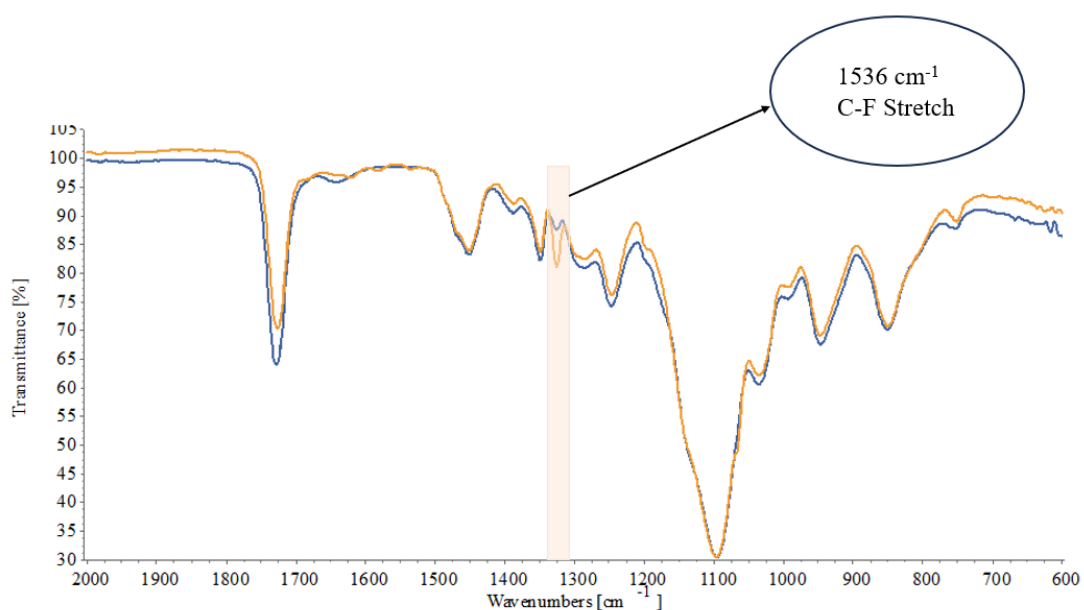


Figure 4.18: FTIR spectra of TFBA-incubated acrylic acid-containing hydrogels (blue) and S-20-TFBA (orange).

4.7. Formation of Microwells and Functionalization of Hydrogels

4.7.1. Formation of Microwells Through Photomask

After the successful fabrication of hydrogel coating, the site-specific formation of amine-reactive domains was achieved using UV light exposure through photomasks. The application of UV irradiation leads to the decomposition of the UV-responsive crosslinker, forming nitroso benzaldehyde groups. This degradation subsequently results in the etching of the surface. By utilizing a photomask, only designated regions on the surface are exposed to UV light, enabling the achievement of site-specific etching and the creation of microwell structures on the hydrogel surface (Figure 4.19).

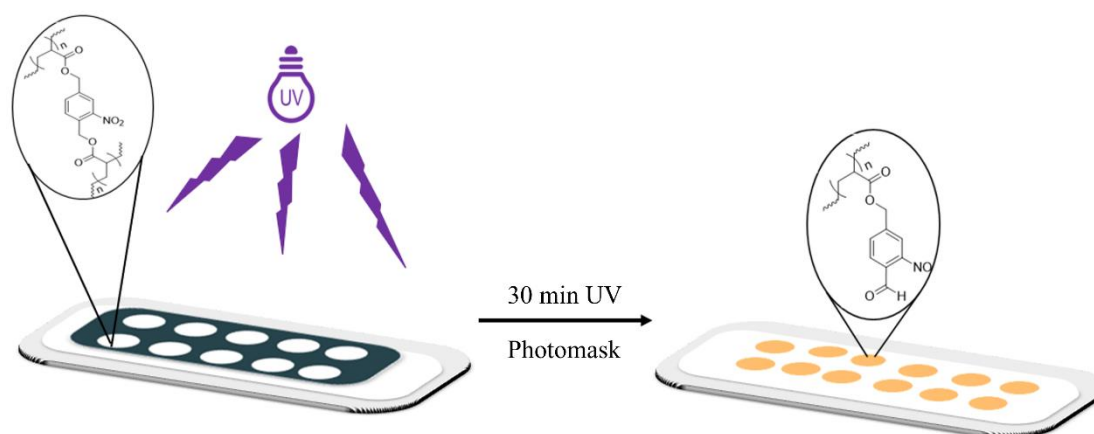


Figure 4.19: Illustration of UV-induced microwell formation through etching on the gel surface.

After UV exposure through round-shaped photomask, hydrogel films were visualized by SEM. As seen in the figures, S-0 hydrogel has no microwell formation after UV exposure due to lack of light-responsive cross-linker. Microwell formation was observed on S-5, S-10 and S-20 hydrogels (Figure 4.20). By using linear shaped photomask, microchannel structures were also prepared, which demonstrates that various types of patterns can be fabricated on these hydrogels by simply changing photomask (Figure 4.21).

Micropatterns can be generated through different ways such as lithographic techniques by using PDMS stamps [57]. Those micropatterned gel structures might not also require photoresponsive groups. However, one drawback might be that after the generation of micropatterns on gel surfaces, then there will be further modifications for biomolecules immobilization. By using ONB groups as crosslinking agents, hydrogels surfaces could be etched by decomposition and various types of patterns could be constructed on gel surfaces UV. Since nitrosobenzaldehyde groups are amine-reactive groups, we showed that this method can be result in reactive microarrays for bioimmobilizations and does not require further modification [62], [63].

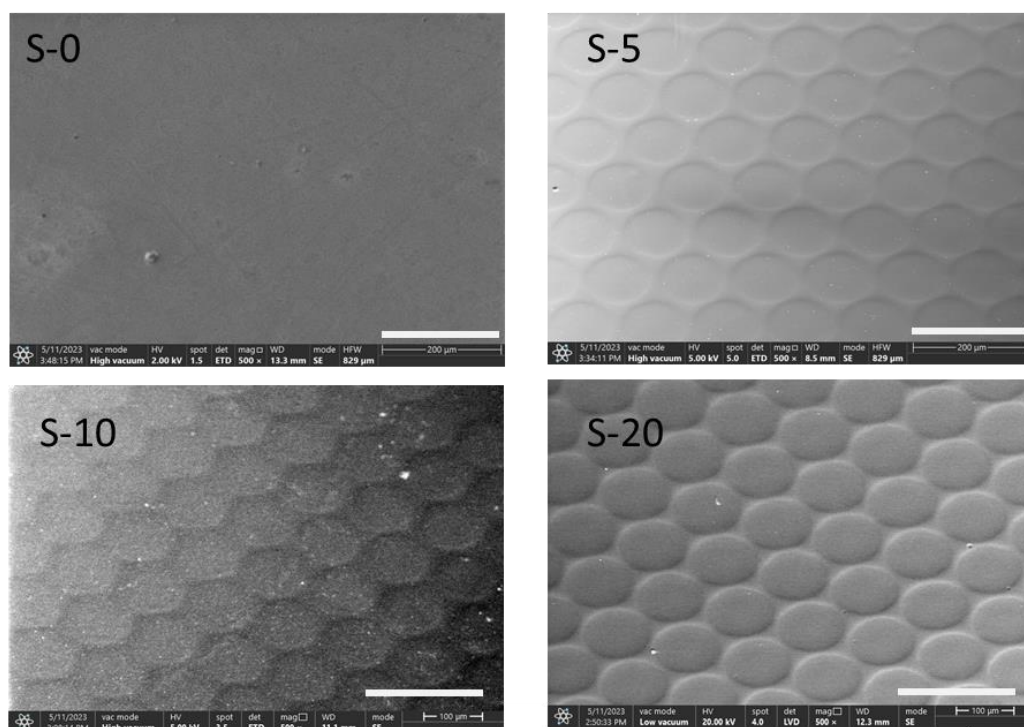


Figure 4.20: SEM images of microwells structures on hydrogel films (scale bar 200 µm).

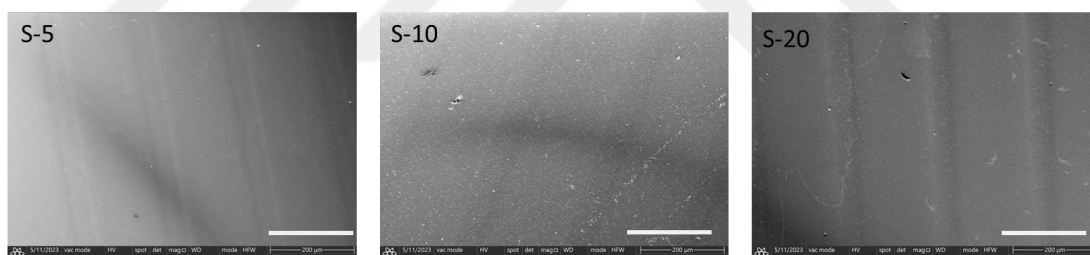


Figure 4.21: SEM images of microchannel structures on hydrogel films (scale bar 200 µm).

4.7.2. Fluorescent Dye Attachment in Amine-Reactive Microwells

Since opaque regions of photomask is assumed to protect hydrogels from UV irradiation, it is expected that only etched parts of hydrogels are activated. Etched parts are inside of microwells or microchannels. To test this, micropatterned hydrogels were incubated in a solution containing fluoresceinamine, and visualized under fluorescence microscope (Figure 4.22.a). No detectable signal was observed in control hydrogel (S-0), while well-defined fluorescent patterns were observed in S-5, S-10, and S-20. As expected, dye molecules conjugated to only inside of channels,

while the outside region remained unreacted. Also, fluorescence intensity increased from S-5 to S-20 showing that by changing ratio of light-responsive group, the amount of immobilized molecule can be altered in the micropatterns (Figure 4.22.b).

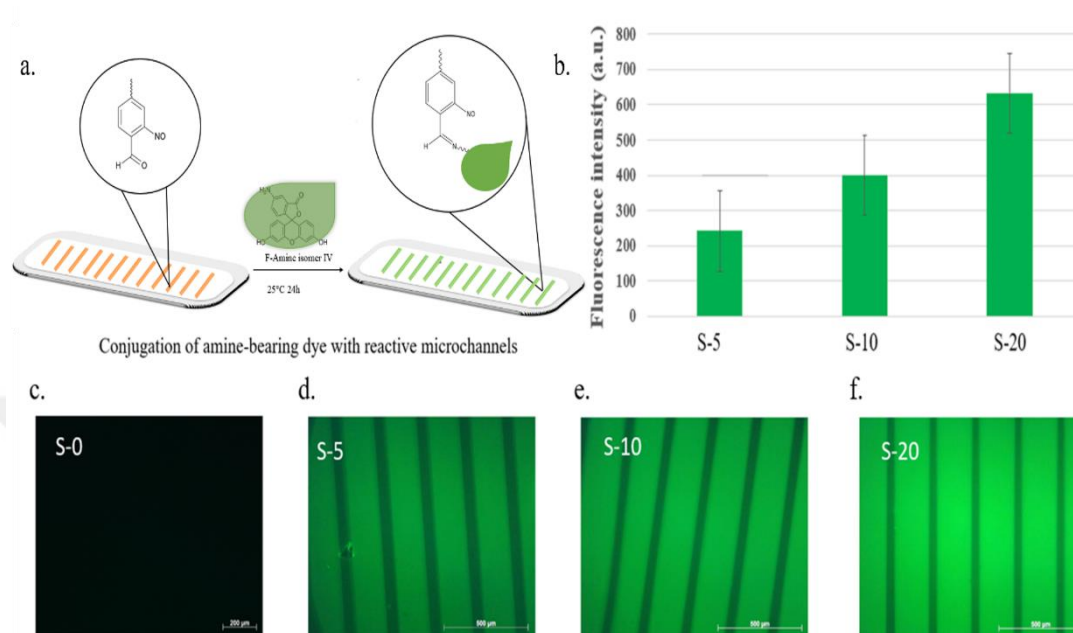


Figure 4.22: a) Illustration of fluorescein amine attachment in microchannels b) Comparison of fluorescence intensity of 3 different gel surfaces containing increasing amount of light-responsive crosslinker. Florescence microscopy images of c) S-0, d) S-5, e) S-10, f) S-10.

4.7.2.a. Fluorescent Dye Attachment on Acrylic Acid Containing Hydrogels

To analyze amine-carboxylic acid linkage, same fluorescent dye attachment protocol was carried out (figure 4.22.a). Fluorescent microscope image shows that there is no fluorescent signal. This result indicates that amine-containing molecules only react with nitrosobenzaldehyde groups (figure 4.23).



Figure 4.23: Fluorescent Microscope images of AA-containing Hydrogels.

4.7.3. Biomolecule (F-BSA) Immobilization in Reactive Microwells

To evaluate the reactivity of the micropatterns toward biomolecules containing amine groups, we employed fluorescently labeled Bovine Serum Albumin (FITC-BSA) for bioimmobilization (Figure 4.24). The patterned surfaces underwent incubation in a solution of FITC-BSA in PBS. After washing with BSA and water, the hydrogel patterns were examined using fluorescence microscopy. The results consistently revealed successful fluorescein amine attachment, with the highest intensity observed at a ratio of 20, followed by ratios of 10 and 5. (Figure 25). Conversely, the control group, which utilized PEGDA instead of NPDMA, did not exhibit any fluorescence. These findings indicate successful biomolecular immobilization onto ONB ester hydrogels. The gradual increase in fluorescence with increasing nitroso benzaldehyde concentration demonstrates that the amount of immobilized biomolecule is directly proportional to the amount of reactive nitroso

benzaldehyde groups. Furthermore, the absence of fluorescence on the outer regions of the microwells, indicates a lack of protein attachment, which demonstrates the antibiofouling property of those regions, which is highly advantageous for biomolecular immobilization-based applications such as bio-sensing (Figure 25).

Taken together, in this section model biomolecule (F-BSA) was successfully conjugated with amine-reactive nitroso benzaldehyde groups. We also showed that conjugation was taken place in reactive microwells, meaning that outside the microwells which UV-mediated etching had not happened showed also significant anti-biofouling activity due to PEG groups in the gel structure. The utilization of a photomask to generate micropatterns containing amine-reactive groups holds immense potential for the immobilization of various protein types and other biomolecules such as enzymes and DNA/RNA. Theoretically, the conjugation of the model biomolecule is believed to occur through a Schiff's base reaction. As Schiff's base reactions are pH-dependent and are more prominent in acidic environments, drug molecules containing $-NH_2$ groups (whether synthetic or biological) can be conjugated, enabling controlled drug release [64]. Furthermore, it has been demonstrated that adjusting the ratio of ONB-containing photoresponsive groups within the gel network has a direct correlation with the amount of attached biomolecules, as depicted in Figure 25/a/b/c. [64]. It has been illustrated that manipulating the ratio of ONB-containing photoresponsive groups within the gel network directly influences the quantity of attached biomolecules, as illustrated in Figure 4.25/a/b/c.

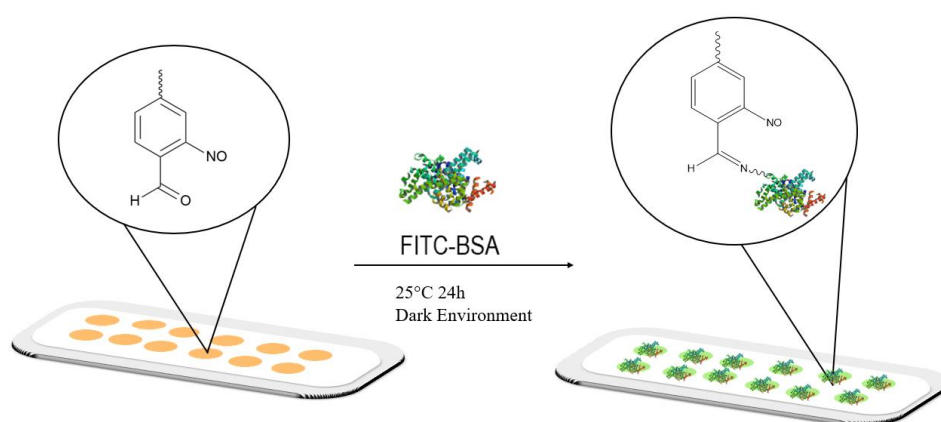


Figure 24: Illustration of model biomolecule attachment in amine-reactive microwells.

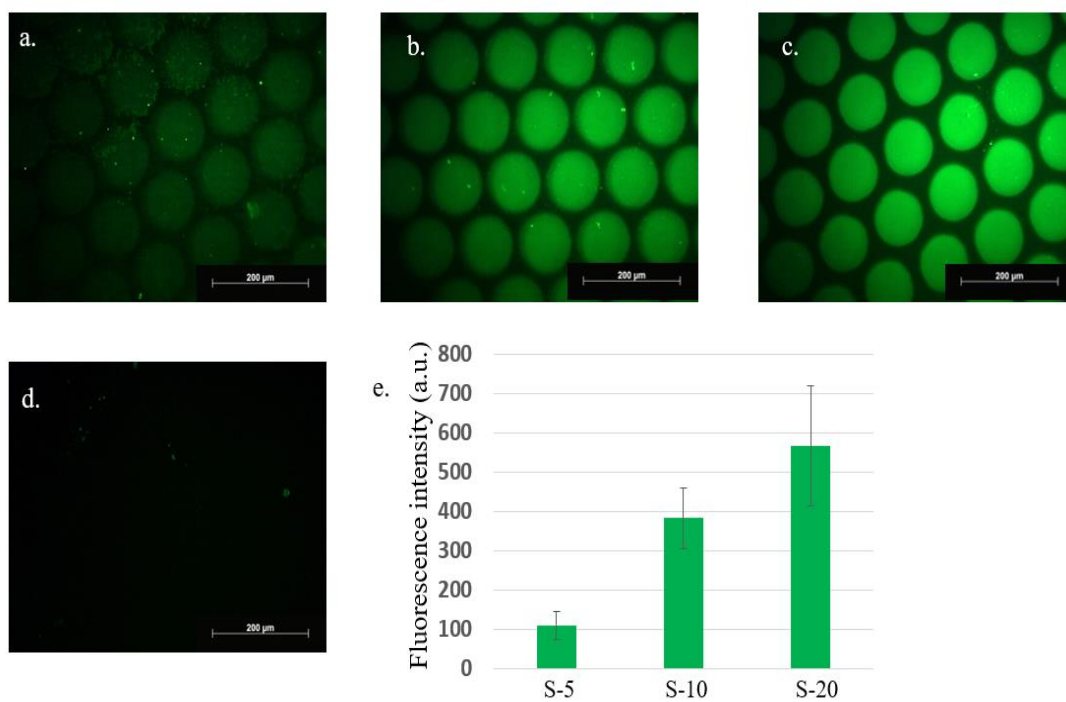


Figure 4.25 Comparison of fluorescence microscope images of 4 different hydrogels. a) S-5, b) S-10, c) S-20 d) S-0. e) Fluorescence intensity comparison of FITC-BSA conjugated microwells.

5. CONCLUSION

In this thesis, amine-reactive microwells or microchannels on PEG based hydrogels were fabricated for biomolecular immobilization. To achieve this PEGMEMA was polymerized with PEGDA and NPDMA as crosslinkers via blue-light mediated photopolymerization. Here NPDMA, possessing a photosensitive ortho-nitrobenzyl ester group, serves as both a crosslinker and exhibits amine-reactive capability upon decomposition. Blue light photopolymerized hydrogels were then exposed to 365 nm UV light. This UV exposure caused structural changes in NPDMA, leading to its decomposition into nitroso benzaldehyde group, which has reactivity towards amine-containing molecules. These alterations in the hydrogel matrices were evaluated and monitored using FTIR spectroscopy. Subsequently, the synthesized gels were exposed to UV light through photomasks, resulting in the formation of microwells and microchannels. The photomask allowed UV light to selectively reach the gel surfaces through the transparent regions, leading to spatially controlled surface etching on the hydrogels. This exposed region was expected to be amine-reactive, while the outside unexposed regions possessed antifouling property.

The amine-reactivity of micro-patterns formed on the hydrogel surface under the influence of UV-light was examined. TFBA, fluoresceinamine, and FITC-BSA were successfully conjugated to the hydrogel surfaces, and this was confirmed using FTIR analysis or fluorescence microscopy imaging. As expected, the target molecules with amine groups were effectively conjugated to the hydrogel surfaces with spatial control. The attachment of amine-bearing molecules to microwells and micro-channels was achieved through photo-generated amine-reactive nitroso benzaldehyde groups. The results obtained in this thesis demonstrate that the synthesized hydrogels have great potential for use in biomolecular immobilization-based applications such as biosensing and tissue engineering.

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Mehmet KIRANŞAL received B.Sc Degree from Molecular Biology and Genetics program (full scholar) at Arel University. During the undergraduate program. He did an internship at Acıbadem University voluntarily, in the Biophysics department. He conducted TUBITAK 2209/A project, named ‘Growing Specific Cell Lines (COS-7 and HEK293) on Hydrogel Scaffold in order to be Used in Tissue Engineering Applications’. After graduation, he started his MSc. Program in the Institute of Biotechnology in Gebze Technical University. He dealt with photoresponsive hydrogels which can be synthesized via photopolymerization, he carried out his thesis study under the supervision of Assistant Professor Tuğçe Nihal GEVREK-CİVAN.

APPENDIX A

CHEMICAL REVIEWS

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Review

Translational Applications of Hydrogels

Santiago Correa, Abigail K. Grosskopf, Hector Lopez Hernandez, Doreen Chan, Anthony C. Yu, Lyndsay M. Stapleton, and Eric A. Appel*



Cite This: *Chem. Rev.* 2021, 121, 11385–11457



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Emerging Fabrication Strategies of Hydrogels and Its Applications

by  Fayaz Ali ^{1,2} ,  Imran Khan ³ ,  Jianmin Chen ^{4,*} ,  Kalsoom Akhtar ¹ ,
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Review

Contact Lens Materials: A Materials Science Perspective

Christopher Stephen Andrew Musgrave ¹  and Fengzhou Fang ^{1,2,*} 

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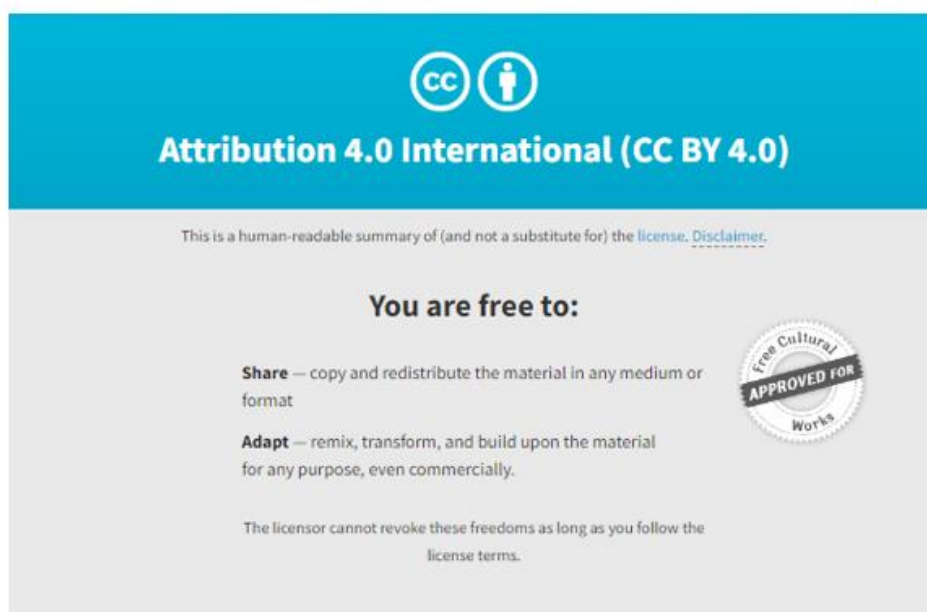


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Review

Hydrogel-Based Adsorbent Material for the Effective Removal of Heavy Metals from Wastewater: A Comprehensive Review

Zenab Darban ^{1,*}, Syed Shahabuddin ^{1,*}, Rama Gaur ^{1,*}, Irfan Ahmad ² and Nanthini Sridewi ^{3,*}

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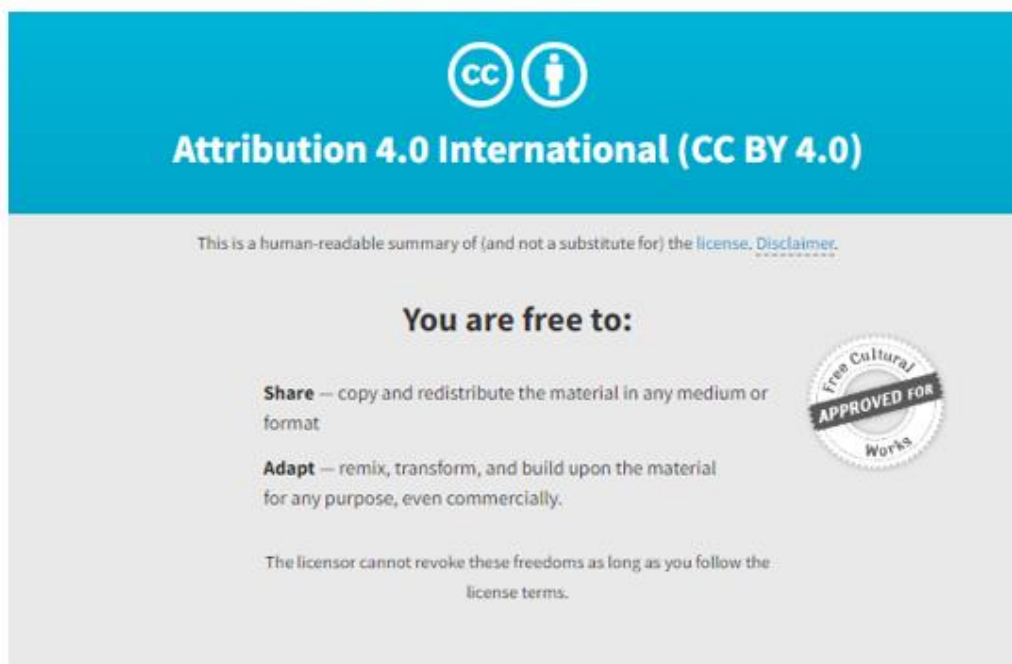


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A Review: Tailor-made Hydrogel Structures (Classifications and Synthesis Parameters)

Author: Reena Singhal, , Kshitij Gupta



Publication: Polymer-Plastics Technology and Materials

Publisher: Taylor & Francis

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Publication: Macromolecular Chemistry and Physics

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Photogenerated Polyelectrolyte Bilayers from an Aqueous-Processible Photoresist for Multicomponent Protein Patterning

Author: Junsang Doh, Darrell J. Irvine

Publication: Journal of the American Chemical Society

Publisher: American Chemical Society

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