

**Visible-Light-Induced Synthesis of Novel pH Responsive
Hybrid Hydrogels for Controlled Drug Delivery**

by

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This is to certify that I have examined this copy of a master's thesis by

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ABSTRACT

In the current research on drug delivery systems hydrogels have become an important topic due to their unique properties such as high water content and sensitivity to the physiological changes in the body. In this study, a novel method was developed for the visible-light-induced synthesis of a pH responsive composite hydrogels for controlled delivery of an anticonvulsant model drug pregabalin (PGB). The pH sensitive layer of the composite hydrogel was based on methacrylic acid grafted with poly(ethylene glycol) tethers P(MAA-g-EG). In addition, the hydrogel was functionalized with hydrophobic domains, through incorporation of crosslinked styrene-butadiene-styrene (SBS) copolymer. Swelling ratios were decreased with the addition of SBS, and as a result high hydrogel crosslink densities were observed. The composites were investigated for controlled release of PGB, and the release was analyzed to describe the mode of transport through the network under neutral and low pH conditions. *In vitro* human fibroblast survival assay and *in vivo* rabbit implantation experiments demonstrated that, these hybrid hydrogels were not toxic and had desirable biocompatibility properties. This is the first report about the synthesis of a pH responsive composite hydrogels via visible light photopolymerization. This technique could be useful for incorporation of molecules with specific functionalities so that sequential molecule delivery in response to specific stimuli could be achieved. This approach can also be used for the design of multifunctional membranes for various tissue engineering applications.

ÖZET

Hidrojeller, yüksek su tutma kapasiteleri ve vücuttaki fizyolojik değişiklere karşı duyarlı olmaları gibi eşsiz özellikleri nedeniyle, ilaç salım sistemlerinin ilgi alanları arasındadır. Bu çalışmada; pH değişimine duyarlı kompozit hidrojel, görünür ışık altında sentezlendiği yeni bir yöntem geliştirilmiştir ve bu hidrojel antikonvülzan model ilaç olarak pregabalinin (PGB) kontrollü salımında kullanılmıştır. Kompozit hidrojin pH'a duyarlı bölümü temel olarak, makromer olarak metakrilik asite bağlı poli(etilen glikol) uçlarından P(MAA-g-EG) oluşmaktadır. Ayrıca, pH'a duyarlı hidrojel fonksiyonel hale getirmek için; hidrofobik bölümler oluşturacak olan çapraz bağlı stiren-bütadien-stiren (SBS) kopolimeri ilave edilmiştir. Şişme oranları, SBS eklenmesi ile azalmıştır ve bunun sonucunda yüksek çapraz bağ yoğunluklarına sahip hidrojeller gözlenmiştir. Nötr ve düşük pH değerlerinde, hidrojellerden PGB'in kontrollü salımı incelenmiş ve salım, hidrojelden taşınma şeklinin tanımlanması için analiz edilmiştir. *In vitro* fibroblast canlılık analizi ve *in vivo* implantasyon deney sonuçları, kompozit hidrojellerin toksik olmadığını ve istenen şekilde biyouyumlu özellikte olduğunu göstermektedir. Bu çalışma; görünür ışıktaki sentezlenen pH'a duyarlı kompozit hidrojellere ait ilk çalışmadır. Bu teknik, özellikle farklı işlevlerdeki moleküllerin yapıya eklenmesinde ve böylece özel uyaranlara cevap olarak ard arda molekül salımında kullanışlı olacaktır. Ayrıca bu yöntem, farklı doku mühendisliği uygulamaları için çok fonksiyonlu membranların tasarlanmasında da kullanılabilir.

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ABBREVIATIONS

DTT	DL-Dithiothreitol
FDA	Food and Drug Administration
GABA	γ -amino-butyric acid
GAD	General anxiety disorder
GBP	Gabapentin
GI	Gastrointestinal
HEMA	Hydroxyethyl methacrylate
IPNs	Interpenetrating networks
LEV	Levetiracetam
LTG	Lamotrigine
MAA	Methacrylic acid
NIPAAm	N-isopropylacrylamide
OXC	Oxcarbazepine
PAA	Poly(acrylic acid)
PBA	Poly(n-butylacrylate)

PDEAEM	Poly(N,N'-diethylaminoethyl methacrylate)
PEGDA	Poly(ethylene glycol) diacrylate
PEG	Poly(ethylene glycol)
PGB	Pregabalin
PMAA	Poly(methacrylic acid)
P(MAA-g-EG)	Methacrylic acid grafted with poly(ethylene glycol)
PMMA	Poly(methyl methacrylate)
SBS	Styrene-butadiene-styrene
SBS-g-HEMA	Hydroxyethyl methacrylate grafted SBS polymer
TEA	Triethanolamine
TGB	Tiagabine
TPM	Topiramate
ZNS	Zonisamid

NOMENCLATURE

C_t	PGB concentration in the release solution at time t
h	Planck constant
M_f	Final PGB mass remaining in the solution
M_0	PGB mass in the stock solution
M_t	Mass released at time t
M_∞	Total weight of PGB released
q	Weight swelling ratio
pKa	Acid dissociation constant
t	Time
V	Total volume of release solution
V_s	Sample volume
W_d	Initial weight of the dried hydrogel
W_s	Weight of the swollen hydrogel
γ_{sv}	Solid-vapor surface tension

γ_{sl}	Solid-liquid surface tension
γ_{lv}	Liquid-vapor surface tension
ν	Frequency

CHAPTER 1

INTRODUCTION

The most significant purpose of the drug delivery systems is to deliver a therapeutic agent into the blood stream with a desirable pharmaceutical effect. Despite significant advances in the development of drug delivery systems, several challenges exist for effective and efficient delivery of drugs related to their physiological properties [1, 2]. Biomolecules such as protein and peptide therapeutics can only remain in the plasma circulation for a short period of time, since they are generally degraded by proteolytic enzymes [3]. As a result, higher dose or number of daily injections of the drug is required for the patient to achieve therapeutic plasma drug concentrations,[4].

Development of functional and responsive polymeric network structures as delivery vehicles for controlled drug delivery is promising for sequential release of drugs in case of a specific stimulus [5]. Polymeric hydrogels, 3D hydrophilic networks, are especially attractive due to their biocompatibility, capacity to retain water, elasticity, softness, and widely usage as drug delivery vehicles [6-8]. Thus, hydrogels have been increasingly used in biomedical and pharmaceutical applications including drug delivery systems [5]. Hydrogels have capacity to deliver therapeutics to a specific site with a stimuli responsive manner, and hence these network systems demonstrate significant potential for different types of biomedical applications [8, 9]. In addition, stimuli sensitive

hydrogels can exhibit favorable physicochemical features depending on their collapsed or swollen states [10, 11]. These properties change prominently in response to alteration in environmental properties such as temperatures, wavelength of light, ionic strength, electric field, magnetic field and pH [9, 12-14]. Specially, pH responsive hydrogels can detect changes in environment pH, and as a result physical characteristics of the network structure such as shape and size can alter [15, 16]. Moreover, pH responsive hydrogels are especially useful for oral administration of the drug along the digestive tract due to the drastically different pH conditions observed at different locations, or for targeted drug delivery into tumor site due to alterations in the acidity of tumor tissue [17-19].

Methacrylic acid grafted with poly(ethylene glycol) P(MAA-g-EG) hydrogels are frequently used as pH sensitive polymeric networks and the responsive nature is because of the existence of ionic groups within the structure. As a weak polyelectrolyte, poly(acrylic acid) (PAA) can accept protons in an acidic environment whereas donate them in a basic environment [17]. As a result of these electrostatic forces, protons transfer from hydrogel to the buffer and hence carboxylic groups become negatively charged and chains repulse. With repulsion, chains extend and hence release of an entrapped drug from the polymeric network occurs at high pH [20]. In the previous studies UV light exposure were used for the synthesis of pH sensitive hydrogels via free radical photopolymerization [17]. Although polymerization of pH sensitive hydrogels under UV light is significant for biomedical applications, biocompatible and responsive hydrogels could be synthesized through visible light exposure. Visible-light-mediated

photopolymerization can induce faster synthesis and less amount of initiator [21]. It is possible to synthesize a hybrid network structure through a visible light initiation mechanism, since drug release from hybrid hydrogel systems is attracting significant attention in the literature [22, 23].

Controlled delivery of anticonvulsant drugs, such as PGB, is important for the treatment of chronic conditions, for example neuropathic pain [24], general anxiety disorder (GAD) [25] and epilepsy [26, 27]. In clinical studies, several second generation anticonvulsant drugs including PGB, have been improved performance against drug resistant epilepsies [28]. Besides clinical use of PGB, extended release of PGB from a drug delivery vehicle has not been taken into account previously [29, 30]. Thus, in this study, a pH responsive hybrid hydrogel system was developed for controlled release of an anticonvulsant model drug, PGB. P(MAA-g-EG) is chosen as hydrophilic and pH sensitive component, where crosslinked SBS was incorporated into this network for hydrophobic function and improved integrity. Slightly hydrophobic gels were synthesized separately, and then added into the pH responsive prepolymer for the synthesis of a composite system. Incorporation of crosslinked SBS in the form of disc or small particles promoted drug release, which can be utilized for a variety of biomedical applications.

In this thesis, chapter 2 presents an overview on recent investigations in stimuli responsive hydrogels and drug delivery systems. Information about pH sensitive P(MAA-g-EG) hydrogels has been provided, and synthesis of SBS incorporated hybrid

hydrogels has been explained in detail. In addition, this chapter includes an overview about the drug delivery methods for controlled release of an anticonvulsant drug PGB.

Chapter 3 includes materials and methods as an experimental part of this thesis. In this chapter, synthesis and characterization of SBS gel, pH responsive hydrogel and hybrid hydrogels were described. Swelling experiments and PGB release experiments were carried out under low and high pH conditions for P(MAA-g-EG) hybrid hydrogels. *In vitro* human fibroblast survival assay, and *in vivo* implantation experiments, important for biocompatibility of the hybrid hydrogels, were explained in this chapter.

Chapter 4 presents the results and discussion of all experiments discussed in Chapter 3. As a final part of this thesis, Chapter 5 summarizes the whole study with a conclusion and also presents future work that can be performed using this developed system.

CHAPTER 2

LITERATURE REVIEW

2.1 DRUG DELIVERY SYSTEMS

The goal of a drug delivery system is to deliver a pharmaceutical agent into the blood and generate a desirable pharmaceutical effect within the biological system. A pharmaceutical agent must be administered with a therapeutic concentration having an impact on a specific site of action and maintained for a particular amount of time. Due to the therapeutic action of the drug, there are several parameters that are used for the desirable administration of the drug. These factors generally consist of the region of the body in which action takes place, the time period of the administration of the drug and the time that the drug is sustained at a desirable therapeutic concentration level. In addition, the choice of a delivery method is significant in terms of the administration route; for instance, oral, transdermal, nasal or parental routes can be selected due to the purpose of the application. Also, physicochemical characteristics and therapeutic features of the drug have important influences on the method of administration [1].

Drug delivery systems are commonly analyzed in two main groups: according to their route of administration, and mechanism of drug release (Figure 2.1).

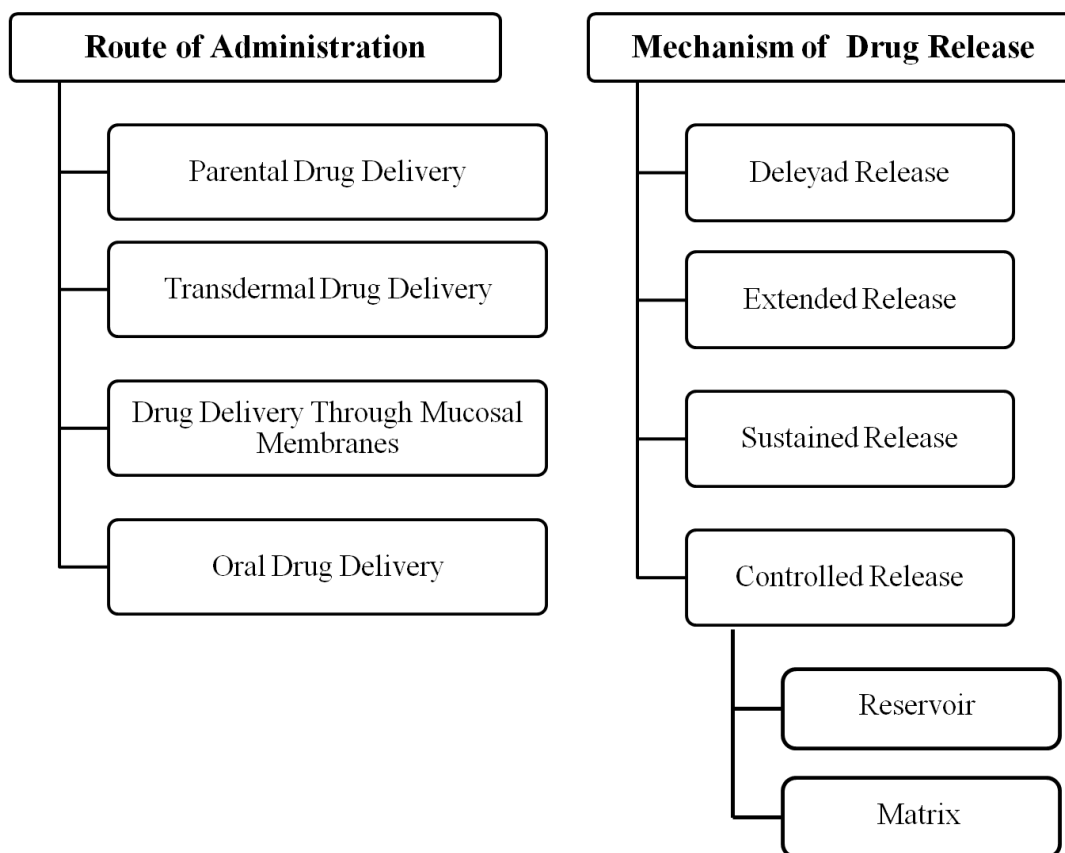


Figure 2.1 Two main groups and subgroups of drug delivery systems.

2.1.1 DRUG DELIVERY SYSTEMS ACCORDING TO THEIR ROUTE OF ADMINISTRATION

2.1.1.1 Parenteral Drug Delivery

In parenteral drug delivery systems, drug is administered into the body either by injection or infusion. Parenteral administration can be sub-classified based on the site of administration, such as intravenous, intramuscular, subcutaneous, intradermal and intraperitoneal administration. In general, aqueous solutions of drug formulations and dispersions of liquid and solid forms are used [31].

2.1.1.2 Transdermal Drug Delivery

Transdermal administration of a drug involves injection of the drug through the skin and into the bloodstream. Although semisolid drug forms are mostly used, liquid dosage forms and solid formulations are also employed [31]. The main idea of the transdermal drug delivery systems is the controlled delivery of therapeutic agents across the skin into the systemic circulation. Polymers and particularly polymeric layers constitute an essential part of the design of transdermal delivery systems. In a transdermal delivery system, there may be a layer where drug is dispersed or dissolved within the polymeric network, such that the system acts as a controlled drug delivery vehicle resulting in release of the drug through the skin [32]. These polymeric layers can also be used as protective coating of the therapeutic agents from a possible external damage.

2.1.1.3 Drug Delivery through Mucosal Membranes

Mucosal membranes such as the one found in small intestine, have special absorption sites. In addition, the gastrointestinal tract is the most important route for the delivery of drugs by oral means. Since gastrointestinal tract is the most important route, oral dosage formulations are usually preferred over the other types of delivery methods. There are also other absorption sites, such as buccal, rectal, vaginal, sublingual mucosa and nasal mucosal membrane [31].

The nasal route can be considered as an alternative site for drug administration.. The intranasal drug delivery system is specifically useful for therapeutic components which are not absorbed through the nasal mucosa because of their chemical properties. In

addition, delivery of a drug using intranasal drug delivery system brings some benefits to the patient, such as lower doses, fewer side effects and quicker attainment to the therapeutic blood level [4].

2.1.1.4 Oral Drug Delivery

Oral drug delivery systems are commonly preferred route for administration of the therapeutic agents.. The oral route is currently employed as a common drug delivery method, since it brings some advantages as; easy administration, patient compliance, and cost effective manufacturing [33]. In addition, oral release mechanisms results in reduced concentrations of the drug in the blood, which decreases the toxicity. On the other hand, achieving a therapeutic blood concentration level takes longer time, and only in over dose uses sustained drug concentrations can be observed. There are other limitations related to the design of the drug delivery system via oral means, for instance, molecular size of the therapeutic agent, charge, pKa, adsorption, solubility in water, toxicity and clinical response [34]. Oral therapeutics such as tablets and capsules, which have instant-release mechanisms, disintegrate quickly in the stomach and get dissolved, are generally absorbed by the small intestine. Here, both release and uptake processes must be considered, where efficiency is based on the physicochemical properties of the therapeutic agent (e.g., solubility and stability) and physiological factors (e.g., passing time of the gastrointestinal tract) [34].

In clinical medicine, oral controlled release forms of drugs have been receiving significant attention. These systems have been developed so that the therapeutic agents

could be administered twice or once a day. In addition, colon targeted drug delivery approaches have become popular for local treatment of the colon or the systemic delivery of drugs which are not well absorbed from the GI tract [34].

2.1.2 DRUG DELIVERY SYSTEMS ACCORDING TO THEIR MECHANISM OF DRUG RELEASE

Drug delivery systems are also classified in two categories: immediate release and modified release mechanisms. In immediate release, after administration of the drug, release occurs immediately. In modified release, drug is released after some time period or for prolonged duration of time. Modification of release systems is a need for enhancement of stability, safety and efficacy of the drug. Furthermore, these modifications promote the therapeutic outcome and support patient compliance [31].

2.1.2.1 Delayed Release

Delayed release forms of drugs provide the release of a drug, and preserve the drug from degradation at low pH condition of the stomach. This protection from the acidic environment delays the release before the drug has entered the small intestine. Generally polymers are preferred for coating of the active drug. In these cases, the polymer coating dissolves slowly with the effect of pH, and the drug can cross over the stomach to the small intestine without any significant loss. After traveling to the high pH environment of the small intestine, the drug release occurs immediately [31].

2.1.2.2 Extended Release

In extended release systems, therapeutic agent is released over a prolonged period of time. The frequency of the drug dose decreases, when the release profile of the drug is extended, Frequent dosing is a problem especially for patients who have chronic diseases, because these patients should take the drugs throughout their life. Extended release systems can be developed based on two main mechanisms: sustained release and controlled release formulations [31].

2.1.2.3 Sustained Release

The drug release rate can be kept over sustained period of time, hence drug release through the GI tract takes place with lower concentration and longer time periods compared to the conditions observed with conventional delivery routes. This sustained release reduces the frequency of dosing which is accomplished by using reservoir systems or matrix systems [31].

2.1.2.4 Controlled Release

Controlled drug release mechanisms provide not only sustained release, but also promotes constant plasma concentrations, which literally means controlled concentration of the drug within systemic circulation. Controlled drug delivery approaches have been considered to adjust both the time of administration and location of pharmaceuticals in the body. [35]. Controlled drug delivery systems have significant benefits over the conventional administration of drugs. These advantages include; decrease in dosage, reduced dosing frequency, advanced patient compliance, consistent

level of drug concentration in blood circulation and diminished toxic effect due to the excess amount of a drug [36]. The progress in potential applications of controlled release systems are based on three crucial parameters: the drug, formulation, and route of administration. The drug and its formulation are both critical, as they can temporarily increase the permeability of barrier membranes which can be unsafe and result in an undesirable patient condition [37].

2.1.2.4.1 Reservoir Delivery Systems

Drug delivery systems have been developed with the use of various types of polymeric materials. Especially hydrophobic and non-degradable polymers have been mostly considered in this area. In reservoir drug delivery devices, there is a silicon elastomer tube which includes a liquid reservoir filled with the drug. This system was discovered by Folkman *et al.* as an example for controlled release of small molecules [38]. The reservoir structure has some benefits, such as constant release rates and long circulation life due to the protection of the drug within the reservoir [35].

2.1.2.4.2 Matrix Delivery Systems

Matrix delivery systems are useful, especially when the release of large molecular weight therapeutic agents from the reservoir is compromised. Reservoir systems are expensive to develop and require series of manufacturing steps. Thus, matrix systems have been considered for the delivery of therapeutic agents, large molecules, or proteins. In order to prepare a matrix delivery system, therapeutic agents are generally dissolved or dispersed in a solid polymer phase [35]. In the current field of interest in

drug delivery systems, hydrogels have gained a considerably extensive interest due to their unique properties. The use of hydrogels as drug delivery vehicles, are promising for both biomedical and pharmaceutical applications [15].

2.2 HYDROGELS: SWELLING CONTROLLED DRUG DELIVERY SYSTEMS

Hydrogels are polymeric networks with hydrophilic groups hence they have a capacity to absorb large amounts of water within their matrix. These 3D polymeric networks comprise crosslinks that are responsible for preventing dissolution of the hydrophilic polymer structures. Hydrogels can absorb water by their hydrophilic groups such as -OH, -COOH, -CONH- and -CONH₂. Due to their high water content, hydrogels have been widely used in various types of applications, such as networks for biomedical studies, materials for tissue engineering constructions, and devices for controlled drug release of therapeutic agents [7, 8, 39, 40].

Hydrogels are elastic, and can present dramatic changes in their network with swelling and deswelling behaviour [15]. Physical characteristics of hydrogels are also similar to natural tissue, in terms of their low interfacial tension, softness, high water capacity and permeability to molecules. Moreover, depending on the application, hydrogels can be designed to be responsive to various stimuli, and can be enzymatically or hydrolytically degradable. Because of these properties, hydrogels can be used as contact lenses, matrices for an implant site, artificial tissues and controlled delivery vehicles [41, 42].

Biocompatibility is an important property, when implantation of hydrogels into the body is considered [15, 43].

Hydrogels can be synthesized by either chemical or physical crosslinking which provide them special mechanical and chemical properties. Chemical crosslinks consist of covalent bonds, whereas physical crosslinks include non-covalent bonds. Chemical crosslinks can be created by free radical polymerization, chemical reaction and high energy irradiation. Chemical crosslinks are stronger than physical bonds due to their higher stronger mechanical properties compared to that of physical bonds. On the other hand, physical crosslinks consist of non-covalent interactions such as hydrophobic interactions, entanglements and hydrogen bonds [44, 45].

In addition, as another type of polymeric materials, cryogels are gel matrices that are synthesized in frozen solutions of monomeric or polymeric precursors. Cryogels have interconnected macropores in their structures and they are great carriers for biomolecules. Cryotropic gelation allows polymeric networks with different morphology compared with gels formed in non-frozen system. Their attracted properties, such as large interconnected pores have been recognized in biotechnological applications [46] For instance, Komarova *et al.* had synthesized thermoresponsive gels and cryogels with incorporated emulsions that both of them were capable of heat-induced collapse. According to their results, thermo-responsive composite gels and cryogels containing entrapped emulsions could be used for the release of lipophilic substances [47].

Various types of hydrogels are favorable in biomedical research due to their desirable properties, such as sensitivity to physiological changes of the environment. These hydrogels are considered as “smart”, where they can respond to a stimulus by changing their properties. Thus, in the previous research, many studies have focused on the development of stimuli-responsive hydrogels and analysis of those responses [48].

2.2.1 STIMULI RESPONSIVE HYDROGELS

Stimuli responsive hydrogels can respond to a special stimulus, which come from the environment, and can change their swelling behavior. Due to their capability of sensing external stimuli, these hydrogels are mostly known as “intelligent materials” in the literature. They can alter their physicochemical properties depending on the stimuli; as a result, large amounts of water can be held or therapeutic agents can be released or both are balanced at equilibrium. Hydrogels can be successful candidates to mimic the biological systems in the body. Stimuli responsive hydrogels can be designed to develop novel drug delivery systems which can respond to physiological changes in healthy or diseased states [11, 49, 50].

In the previous studies, various strategies have been used to design responsive hydrogels [Figure 2.2]. These intelligent hydrogels have been categorized depending on the stimuli, and these stimuli can be either internal (e.g. pathological and physiological) or external (e.g. physical stimuli). Thus, the stimuli can be physical or chemical/biochemical type. As shown in Figure 2.3 the physical stimuli consists of

light, heat, pressure, electric field or magnetic field, whereas the chemical stimuli contains ions, pH, redox potential and specific molecules, such as enzyme [12, 51].

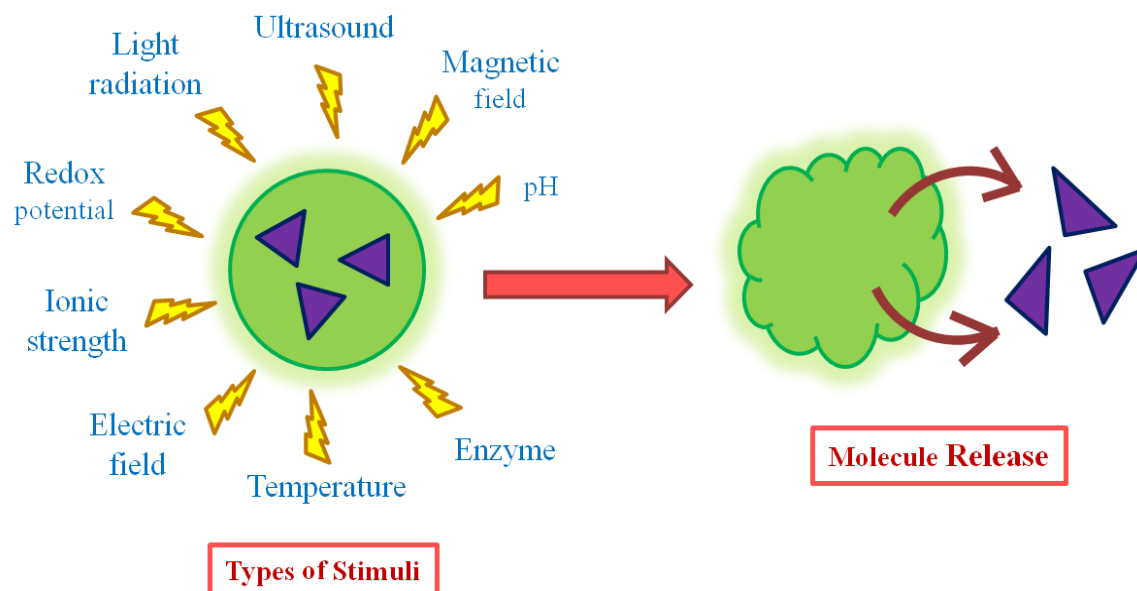


Figure 2.2 Schematic representation of types of stimuli that cause alterations in polymeric material structure.

2.2.1.1 Chemical and Biochemical Stimuli

Chemical and biological stimuli occur as a result of pH changes, antigen-antibody interactions or enzymatic expressions in diseased tissues. For instance, the pH level between the extracellular and intracellular environments is considerably influenced by pathological conditions [12]. Furthermore, the extracellular pH of tumor environment is slightly acidic (approximately pH: 6-7) compared to the pH: 7.4 of the systemic circulation [52].

2.2.1.2 Physical Stimuli

Physical stimuli are classified according to the type of stimuli, and consist of light, pressure, temperature, magnetic and electrical fields. As an example, thermo-responsive polymeric materials have been considered as drug delivery vehicles where the surrounding temperature is changed. Light is another external stimuli which can be controlled easily. Thus, photo-responsive polymers are activated by light that applied at specific frequencies [12].

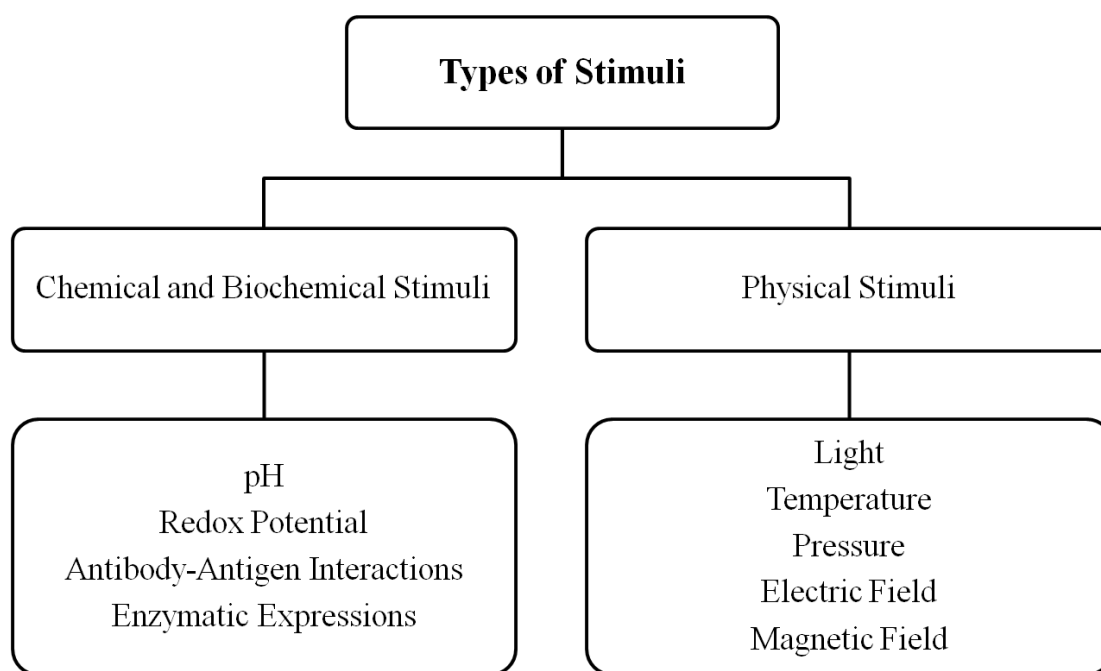


Figure 2.3 Schematic representation of the types of stimuli.

2.2.2 PH RESPONSIVE HYDROGELS

pH responsive hydrogels are designed considering shifts in pH values of various body sites, such as gastrointestinal tract and vagina [15, 16]. pH changes through the gastrointestinal tract, between a large range of pH values, such as 1-2 for a gastric pH, and 7-8 for an intestinal tract. These major differences have been considered for pH dependent release in designing pH responsive drug delivery systems. These pH responsive scaffolds release therapeutic substance as a result of pH alterations in the environment [11].

All types of pH responsive polymeric materials include either pendant acidic or basic groups in their structure. Carboxylic acid represents common example of a pendant acidic group, while ammonium salt is an example of basic group. These functional groups allow polymeric material to accept or release protons in response to shifts in the external pH [51, 53].

Polymers are defined as polyelectrolytes when ionizable groups are retained in their structure. As an example, the structures of an anionic and a cationic polyelectrolytes and the ionization based on pH are shown in Figure 2.4. For instance, Poly(acrylic acid) (PAA) ionized at a high pH environment; however, poly(N,N'-diethylaminoethyl methacrylate) (PDEAEM) ionized at low pH values. Due to the pH dependent ionization process, an anionic polyelectrolyte such as PAA dissolve further at high pH, whereas a cationic polyelectrolyte PDEAEM dissolve at low pH condition [51]. In addition, pH responsive properties of hydrogels are influenced by the nature of the

ionizable groups, crosslinking density and hydrophobicity of the polymer. The feature of an ionizable group (e.g. acidic or basic) has an effect on swelling ratio of hydrogels, while crosslinking density alters the permeability of pH responsive hydrogel scaffolds. Hydrophobicity has a negative effect on the permeability of the hydrogels as well; however, hydrophobicity can be reduced by using copolymerization reaction of hydrophilic and hydrophobic monomers [41].

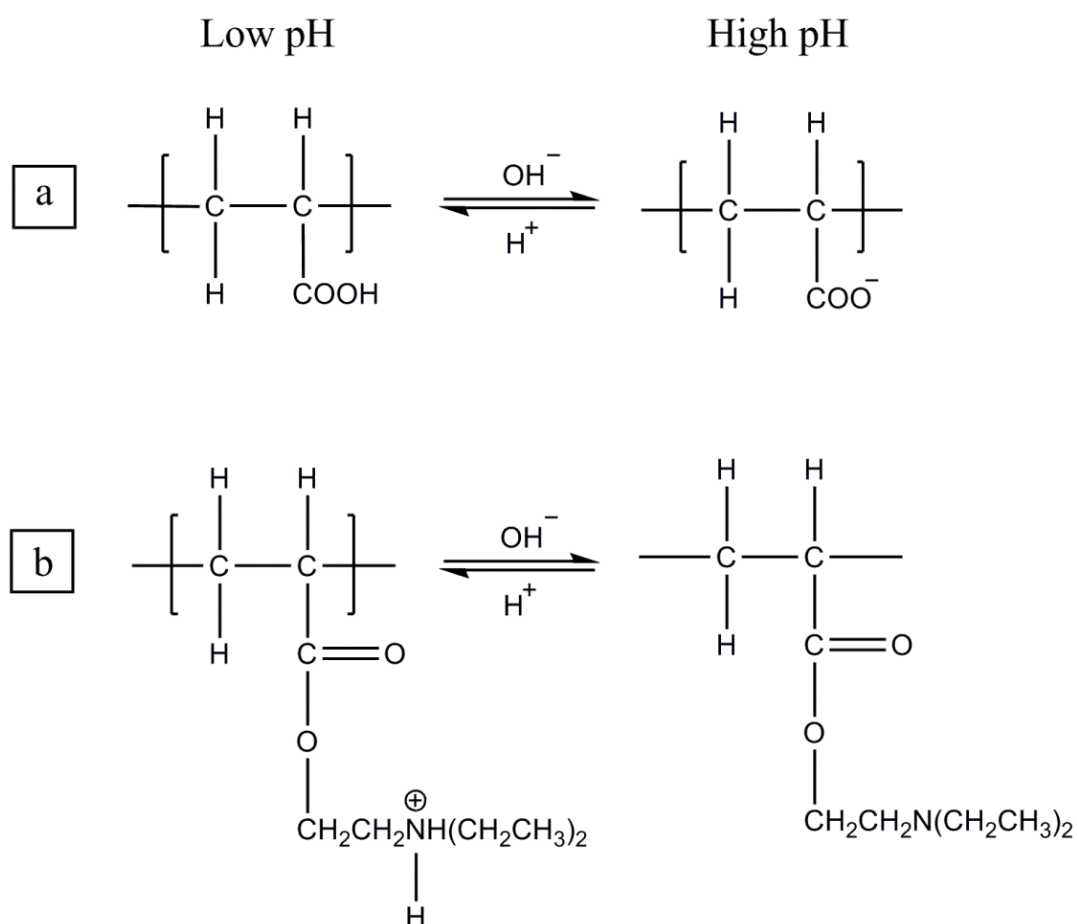


Figure 2.4 The structure and the ionization of (a) an anionic (PAA) polyelectrolyte, and (b) a cationic (PDEAEM) polyelectrolyte.

Polymer chains with ionizable groups have higher swelling ratios than that of any other type of non-electrolyte hydrogel. The swelling ratio of a hydrogel scaffold is based on the electrostatic repulsive forces that come from the charges of the polymeric chain. The amount of swelling can change as a result of any condition that decreases electrostatic repulsion, for example pH or ionic strength [51, 54]. The ionization of pendant groups of anionic hydrogels occurs above the pKa value of the network, whereas the pendant groups of cationic hydrogels are ionized below the pKa value of the network. In both conditions, the ionization process guides swelling of the hydrogel at a specific pH range. For anionic hydrogels, this pH range will be higher than the pKa of the polymer, and for cationic hydrogels pH range will be lower than the pKa [15]. The swelling behavior of ionic hydrogels is different in acidic and alkaline buffer solutions, as shown in Figure 2.5.

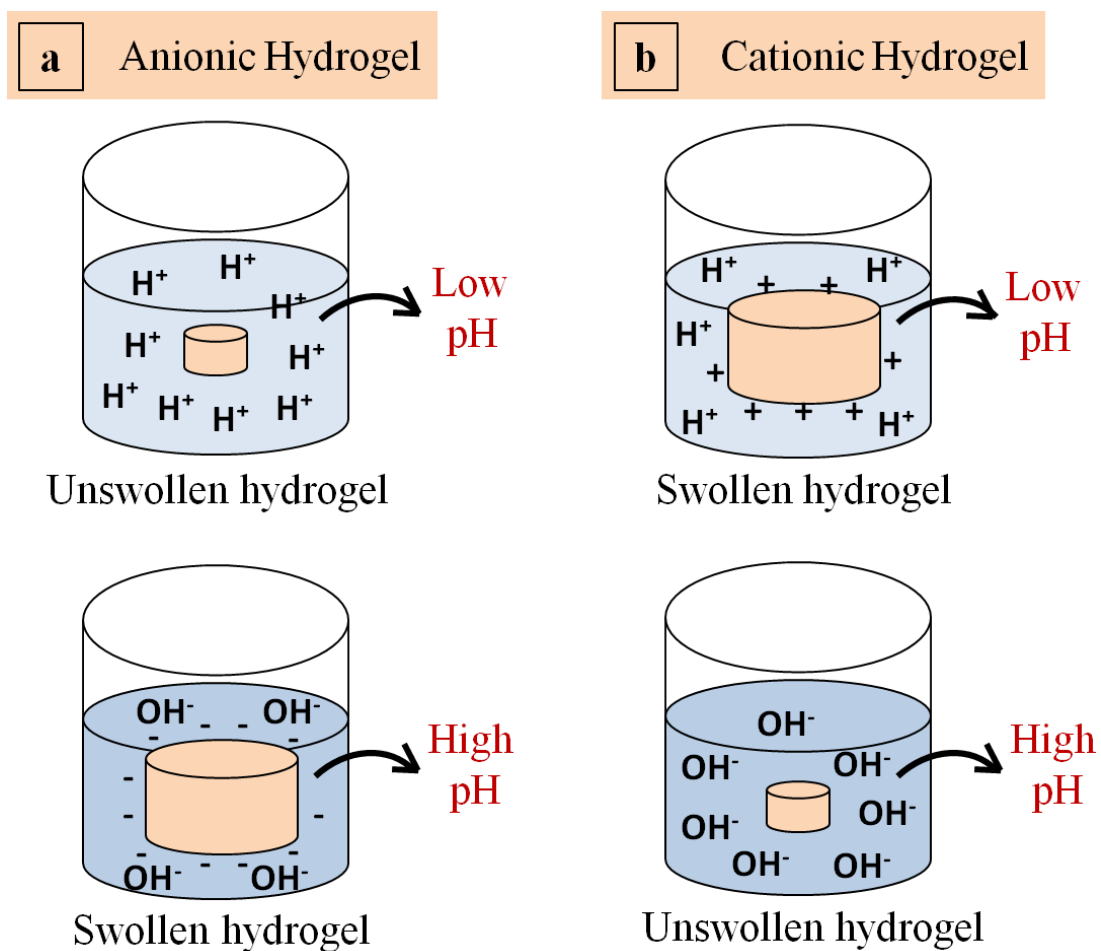


Figure 2.5 pH sensitive swelling behavior of (a) anionic and (b) cationic hydrogels.

In the literature, neutral comonomers (e.g. methyl methacrylate, maleic anhydride) are commonly used to regulate the swelling ratio and pH sensitive property of hydrogels. Depending on the comonomer, the hydrophobicity of the chain can be different and this may cause alterations in the pH responsive behaviour of the scaffold [55, 56].

2.2.2.1 P(MAA-g-EG) Hydrogels

P(MAA-g-EG) hydrogels are commonly used for the design of pH responsive polymeric networks. The responsive nature is due to the presence of ionic groups within the network. Poly(acrylic acid), a weak acidic polyelectrolyte, can accept protons in an acidic environment and donate protons in a basic environment [57].

At high pH values, protons are transferred from hydrogel to the buffer solution, which causes carboxylic groups to become negatively charged and repulse chains. Repulsion causes chain extension in the hydrogel structure at a high pH, and high swelling, which is followed by the release of entrapped drug from the hydrogel network towards the surrounding medium [20].

Peppas *et al.* designed hydrogels with pH responsive properties by using poly(methacrylic acid) (PMAA) grafted with poly(ethylene glycol) (PEG) [58]. The acidic protons of the carboxyl groups of methacrylic acid (MAA) react with the oxygen of PEG by hydrogen bonds at low pH values. The complexation occurs between MAA and PEG, and hence the hydrogels remain in collapsed state. On the other hand, carboxyl groups of MAA are ionized at high pH environment, and swelling takes place by decomplexation [51].

2.2.2.1.1 Formation of P(MAA-g-EG) Hydrogels

pH sensitive P(MAA-g-EG) hydrogels have been commonly synthesized by free radical photopolymerization using UV light where Irgacure has been used as a photoinitiator, as is shown in Figure 2.6 [17]. For example, Schoener *et al.* used UV light mediated polymerization to synthesize both P(MAA-g-EG) hydrogels with poly(methyl methacrylate) (PMMA) nanoparticles [18], amphiphilic interpenetrating networks (IPNs) of P(MAA-g-EG), and poly(n-butyl acrylate) PBA [59].

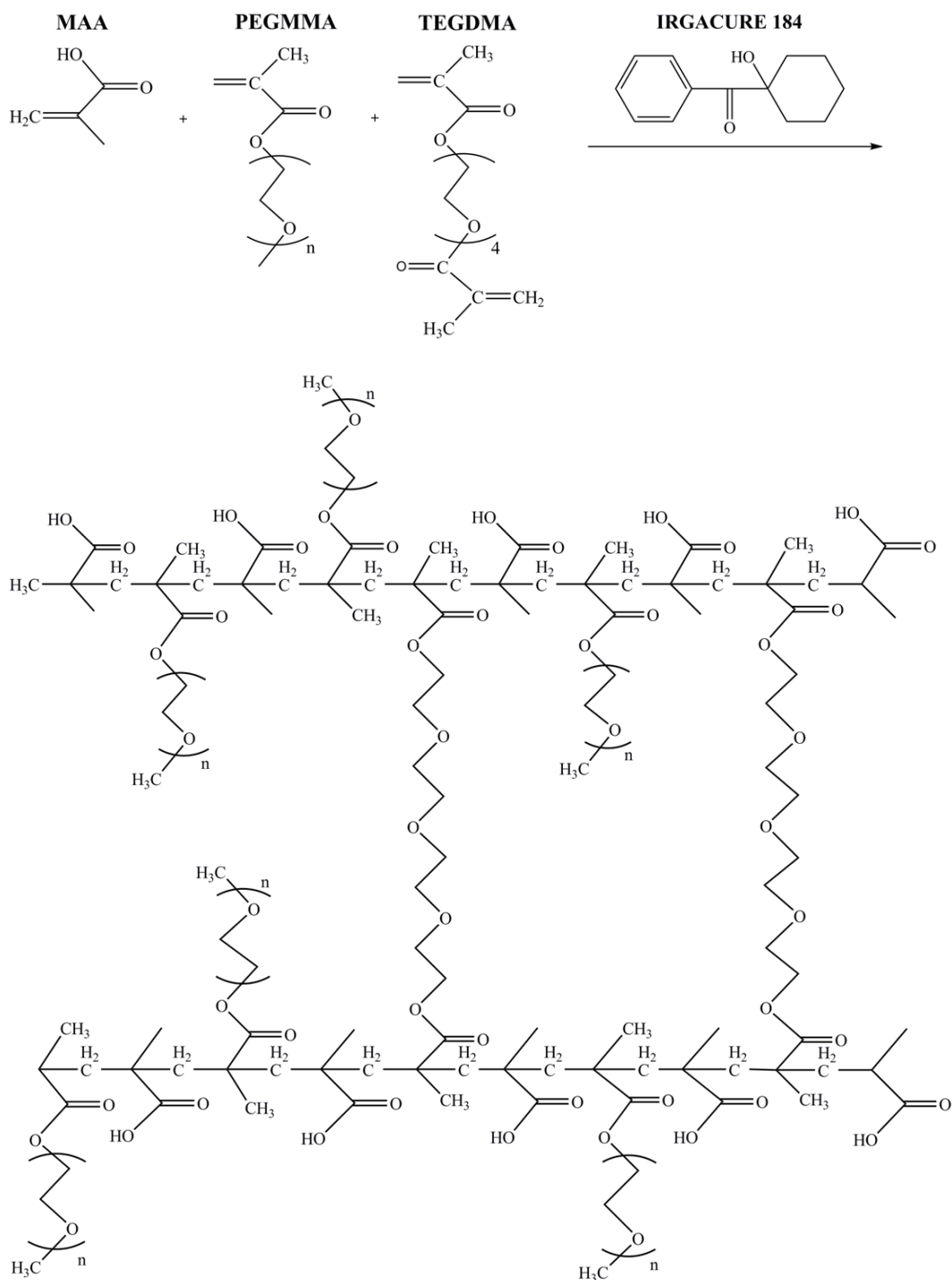


Figure 2.6 Synthesis of P(MAA-g-EG) hydrogels through free radical photopolymerization via UV light.

Although synthesis of P(MAA-g-EG) hydrogels under UV light is significant for biomedical applications, biocompatible and responsive hydrogels could also be obtained through visible light exposure. In addition, visible-light-induced photopolymerization process can result in faster polymerization reaction, where less amount of initiator can be used compared to UV light-induced synthesis condition [21]. Based on the previous studies of Kizilel *et al.* [60, 61], PEG diacrylate hydrogels can be synthesized by visible-light-induced photopolymerization. In this study, P(MAA-g-EG) hydrogel synthesis was carried out by using the same photoinitiation mechanism using eosin Y and TEA as a photoinitiator, and a co-initiator respectively. The purpose of choosing eosin as a photoinitiator, is based on the convenience of its spectral properties with an argon ion laser. As a co-initiator, TEA acts as an electron and a proton donor, which result in the formation of alpha-amino radical. This radical is the primary radical to initiate polymerization and further crosslinking reactions. The details of photoinitiation process, which consists of irradiation by visible light (514 nm wavelength), is shown in Figure 2.7. [60]. Thus, the main difference between current visible light and previous UV light polymerized pH sensitive hydrogels is the addition of eosin Y and TEA instead of Irgacure.

polymeric system [63]. Such composite hydrogel systems retain both hydrophilic and hydrophobic properties together. For example, Men *et al.* used cationic nanoparticles in combination with thermo-sensitive Pluronic hydrogel, and investigated the delivery of hydrophobic anticancer drugs from this system [64]. In another study, a model drug ketoprofen was released from hydrophobic silica aerogel coated within PEG hydrogel [65]. The term hybrid or composite hydrogels refer to hydrogel systems in combination with polymers [66], or a network that includes particulate systems [42]. As a separate approach for creating hybrid structures, grafting of hydrophilic polymers with hydrophobic polymer chain has been used. Even though these hydrogels had excellent properties due to their similarity with human tissue, and great tissue compatibility, they showed poor mechanical properties upon swelling [67]. To address this problem, Hsiue *et al.* have developed hydroxyethyl methacrylate (HEMA) grafted SBS polymer, and observed superior mechanical properties of SBS-g-HEMA compared to that of poly(HEMA) [68]. Lee *et al.* also investigated grafting of N-isopropylacrylamide (NIPAAm) on SBS to increase water absorption, and obtain networks with thermo-sensitive property [69]. Thus, functionalization of biomaterials with other polymeric materials, and development of hybrid systems have potential for biomedical applications.

2.2.3.1 Formation of SBS Gel Incorporated Hybrid Hydrogels

(SBS) block copolymer is a typical thermoplastic elastomer, which contains glassy and elastomeric domains together. Figure 2.8 demonstrates the structure of SBS triblock copolymer. SBS consists of great properties such as high extensibility, elastic recovery and resistance to water [70]. Crosslinking of triblock SBS polymer can be achieved through thiol-ene chemistry and this photoinitiated thiol-ene polymerization progresses by a step-growth addition mechanism.

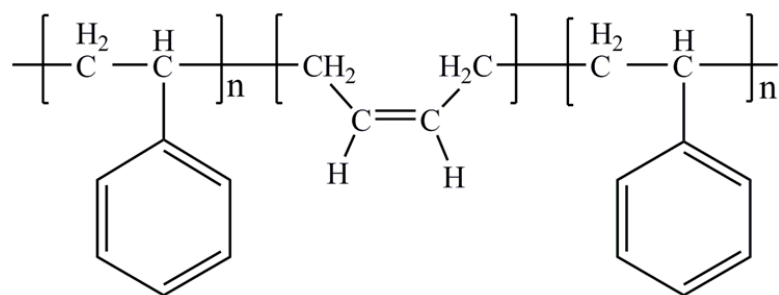


Figure 2.8 Structure of SBS triblock copolymer.

The step-growth mechanism is generated by a chain transfer reaction and includes the thiyl radicals which are shown as $\text{RS}^\bullet$ (Figure 2.9). The thiyl radicals, generated with photoinitiation mechanism, react with vinyl and the 2-butene double bonds, as a result a chemically crosslinked network is produced. As shown in the study by Decker *et al.*, the thiol-ene polymerization was an effective approach for crosslinking of SBS [70].

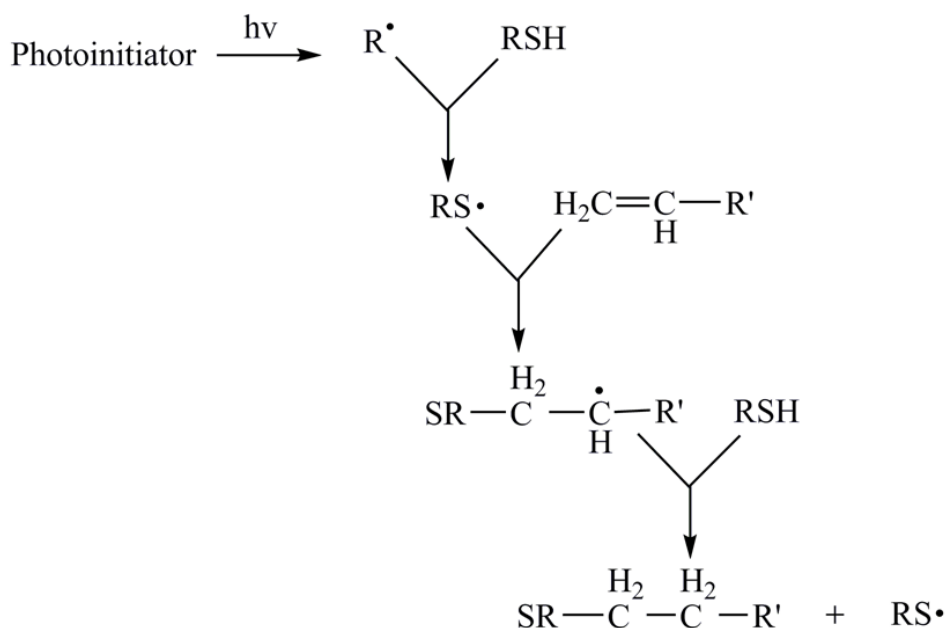


Figure 2.9 The step-growth mechanism of the thiol-ene polymerization.

As demonstrated in Figure 2.9, crosslinked SBS polymer was synthesized by allowing the reaction of thiol radicals with the double bonds present in the polybutadiene units of SBS. With thiol-ene chemistry, the formation of radicals can be initiated by various methods such as UV, thermal and visible light [71].

As a crosslinker, DL-Dithiothreitol (DTT) [Figure 2.10] which is a bi-functional thiol, could be used for thiol-ene crosslinking reaction as demonstrated in Figure 2.11. In order to synthesize crosslinked SBS, DTT was used as a crosslinker, eosin Y as a photoinitiator and TEA as an accelerator. As a result, this thiol-ene photopolymerization reaction occurred by visible-light-curing (514 nm wavelength) instead of UV light.

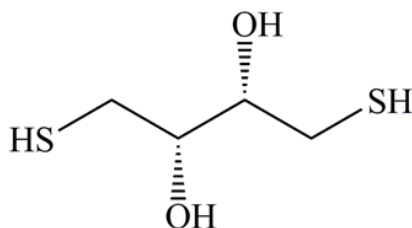


Figure 2.10 The structure of DL-Dithiothreitol (DTT).

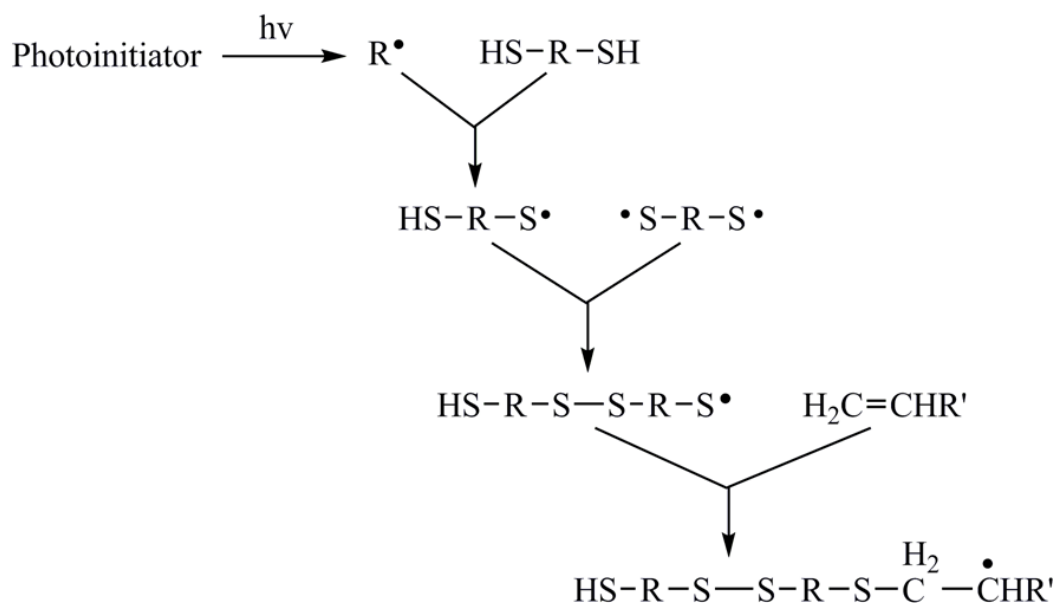


Figure 2.11 Photoinitiated thiol-ene reaction with addition of DTT as a crosslinker.

2.3 CONTROLLED DELIVERY OF AN ANTICONVULSANT DRUG

In clinical studies, a set of second generation anticonvulsant drugs including gabapentin (GBP), lamotrigine (LTG), levetiracetam (LEV), oxcarbazepine (OXC), pregabalin (PGB), tiagabine (TGB), topiramate (TPM) and zonisamide (ZNS) have been shown to provide minimal side effects and improved performance especially against drug resistant epilepsies [28]. Pregabalin has a significant role in the central nervous system,

which acts to generate an inhibitory modulation of neuronal excitability [24]. Controlled delivery of anticonvulsant agents, especially PGB is crucial for the management of chronic conditions, such as neuropathic pain [24], general anxiety disorder (GAD) [25] and epilepsy [26, 27]. Neuropathic pain can be defined as “pain that occurs as a result of a lesion or disease affecting the neuronal system”, and this pain is characterized by alterations in the function and chemistry of neurons [24]. Thus, in neuropathic pain, over excitation of neurons occur, which results in neuron damage. Even though several neuropathic pain treatments have not been approved by Food and Drug Administration (FDA), an anticonvulsant PGB is one of the unique FDA approved medication for the treatment of pain for diabetic peripheral neuropathy [72].

General anxiety disorder (GAD) is a chronic and disabling condition, which is characterized by uncontrollable and excessive worry, and it has approximately 12 month prevalence [25]. Although various treatment options exist for GAD, PGB has been considered as a first-line treatment option in the guidelines of the World Federation of Societies of Biological Psychiatry [73].

Epilepsy is a group of long-term neurological disorders which potentially compromises life-span. Along with the anticonvulsant drug therapy, epilepsy usually becomes seizure free in patients. The main purpose of an anticonvulsant drug therapy is to reduce the possibility of a seizure due to serious deficiency in seizure control in epileptic patients [27]. Furthermore, treatment with anticonvulsant drugs is strongly recommended for the patients diagnosed with epilepsy whose seizures might cause serious morbidity [74].

Some preclinical studies of PGB had shown that, it has great potency as an analgesic and anticonvulsant activity for epileptic seizures [75]. Controlled release of PGB from a specific stimuli responsive hydrogel system becomes a significant method for the treatment of these three chronic disorders. According to our knowledge, extended release of PGB from a delivery vehicle has not been studied in previously.

2.3.1 PREGABALIN AS A MODEL DRUG

In 2004, PGB was approved in Europe, for the treatment of peripheral neuropathic pain, and then was approved by US Food and Drug Administration (FDA) for diabetic peripheral neuropathy. PGB [Figure 2.12] is an alkylated analogue of γ -amino-butyric acid (GABA), which is the primary inhibitory neurotransmitter in the central nervous system Figure 2.12 [76]. Although, PGB is a structural analogue of GABA, it does not interact with GABA, or GABA receptors, and it does not compromise the uptake of GABA [77].

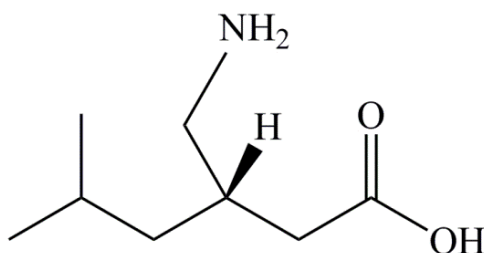


Figure 2.12 The structure of Pregabalin (PGB).

PGB binds with high affinity to the α_2 - δ protein subunit of the presynaptic neuron's voltage gated calcium channels [78, 79]. With binding of PGB to the α_2 - δ subunit, presynaptic calcium channels are closed and calcium influx is decreased at nerve

terminals, as is demonstrated in Figure 2.13. As a result, the release of various excitatory neurotransmitters, such as glutamate, and noradrenaline are reduced. With decreasing in release of excitatory neurotransmitters, the “hyper-excited” neurons return to a “normal” physiologic state [76, 78-80]. PGB only regulates the activity of over-excited neurons. Since the activity of PGB is state dependent, PGB is a unique therapeutic choice for the treatment of epilepsy [81, 82].

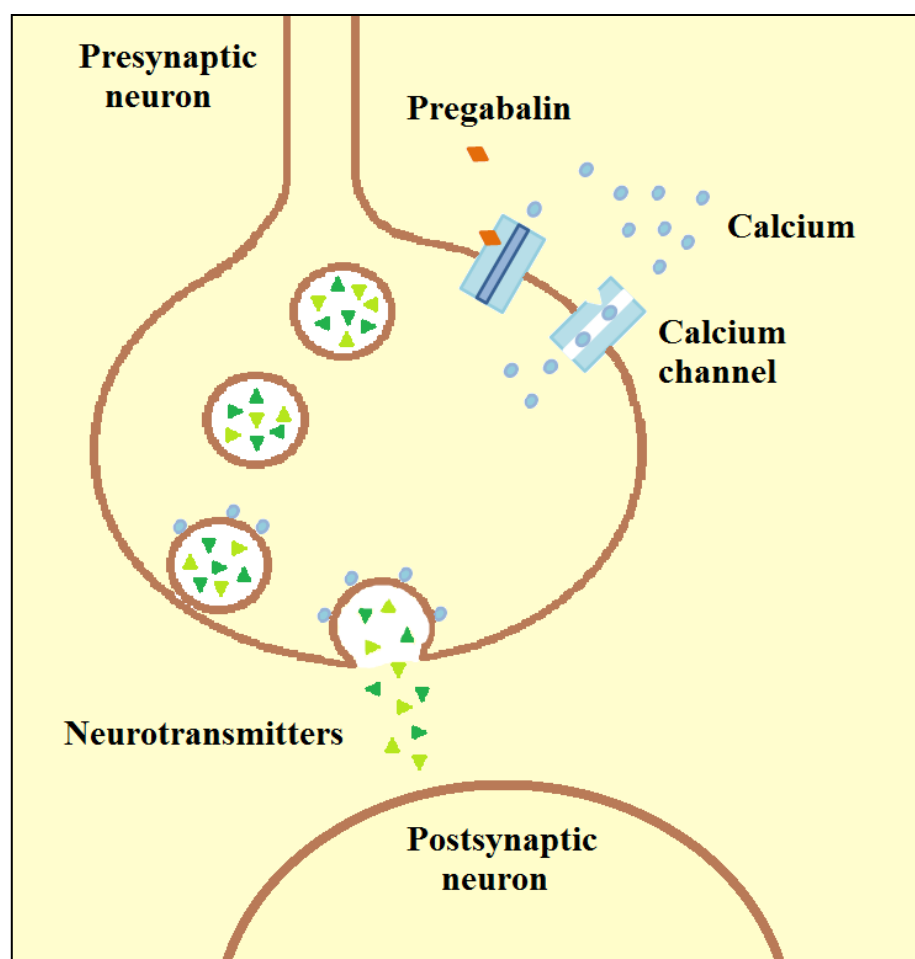


Figure 2.13 Mechanism of action of the pregabalin in the central nervous system.

In addition, among the second generation anticonvulsant agents, PGB has attracted attention due to its extensive use for the treatment of GAD in adults [25]. Despite extensive clinical studies about PGB, controlled and long term controlled release of PGB for inhibition of seizures has not been considered previously [29, 30]. PGB has been considered as a suitable representative drug of these second generation anticonvulsants for the development of a drug delivery vehicle for its controlled release.

Clinical pharmacology studies demonstrate that, single-dose tolerance of PGB is 1 to 300 mg, whereas multiple-dose tolerance of PGB is 25, 100, 200, 300 mg doses for every 8 hours and 300 mg doses for every 12 h [83]. Available formulations of PGB capsules are 25, 50, 75, 100, 150, 200, 225 and 300 mg and usual dosage for adults ranges between 150-600 mg/day. The important consideration is the maintenance of this range in two or three equally divided doses [80]. Thus, controlled release of PGB could be achieved with a single-dose in one day, with the use of a pH responsive hybrid hydrogel system, such as the one developed in this study.

CHAPTER 3

EXPERIMENTAL PART

3.1 Materials

Poly(ethylene glycol) monomethyl ether monomethacrylate (PEGMMA; 1000 g/mol), methacrylic acid (MAA) and tetraethylene glycol dimethacrylate (TEGDMA) were purchased from Polysciences Inc. (Warrington, PA). 1-hydroxycyclohexyl-phenyl ketone (Irgacure 184), DL-Dithiothreitol (DTT), eosin Y, 1% Penicillin/Streptomycin, 2mM L-Glutamin and ethanol (99.8 %) were purchased from Sigma-Aldrich. Triethanolamine (TEA) and Cyclohexanone (99.9 %) were purchased from Merck (Darmstadt, Germany). Styrene-butadiene-styrene block copolymer (SBS D1101, S:B wt fraction: 70:30) was purchased from Kraton, and 3,3-dimethylglutaric acid (DMGA) was purchased from Fisher Scientific (New Jersey, USA). Dulbecco Modified Eagle's Medium (DMEM) was purchased from Life Technologies (Paisley, UK) and 10% fetal bovine serum (FBS, heat inactivated) was purchased from Biological Industry. Phosphate-buffered saline (PBS) tablets were purchased from Amresco (Solon, Ohio). PGB was kindly supplied by Abdi İbrahim (Pharmaceutical Company, Istanbul, Turkey), in powder form. All chemicals were used without any prior process except MAA which was passed through a column to remove the inhibitor hydroquinone.

3.2 Methods

3.2.1 Synthesis

3.2.1.1 Synthesis of Crosslinked SBS Gel

SBS prepolymer solution was prepared with 5.5 wt/vol % SBS, 5 wt % DTT, 225 mM TEA, and 0.15 mM eosin Y in cyclohexanone. This prepolymer solution was mixed and exposed to visible light at 514 nm with a flux of 10 mW/cm² using an argon ion laser (Coherent Inc, Santa Clara, CA) for 40 min. As a result, SBS polymer discs were formed, and then they were washed in cyclohexanone for several days and were dried in fume hood. After drying, crosslinked SBS was added into P(MAA-g-EG) prepolymer solution in the form of disc with 3 mm diameter or 7 shredded particles with about 1 mm diameter.

3.2.1.2 Synthesis of Hybrid Hydrogels

The P(MAA-g-EG) prepolymer solution includes of 3.6 g of MAA and 2.0 g of PEGMMA. For the case of photopolymerization under UV light, the cross-linking agent TEGDMA was added in the amount of 0.5 mol % of the total monomer, and the photoinitiator Irgacure 184 was added 1.0 wt % of total monomer. The monomer solution was diluted with 50:50 (w/w) solution of ethanol and water mixture. Next, both crosslinked SBS polymer discs and crosslinked SBS shredded particles with total particle mass of 4 mg were added into the prepolymer solution. After shaking at 120 rpm for 24 h, the mixture was exposed to UV light (Output wavelength of Ti:sapphire pumped optical parametric amplifier (OPA) was tuned to 300 nm with 30 mW power

for 30 minutes). The two different types of composite gels were washed several days in distilled water and freeze-dried (Labconco, FreeZone 2.5 liter Benchtop Freeze Dry System).

For the case of synthesis under visible light, the P(MAA-g-EG) prepolymer solution consisted of 3.6 g of MAA, 2.0 g of PEGMMA, and 0.5 mol % TEGDMA. Next, 225 mM TEA and 0.03 mM eosin Y were added into the prepolymer solution, which included 50:50 (w/w) solution of ethanol and water mixture. Then, crosslinked SBS in the form of disc and shredded particles were added into the prepolymer solution, shaken at 120 rpm for 24 h. Next, the solution was exposed to visible light (514 nm and 7.5 mW/cm²) using argon laser for 20 min. Finally, hybrid hydrogels were rinsed with distilled water for several days and freeze-dried. This whole procedure is shown in Figure 3.1.

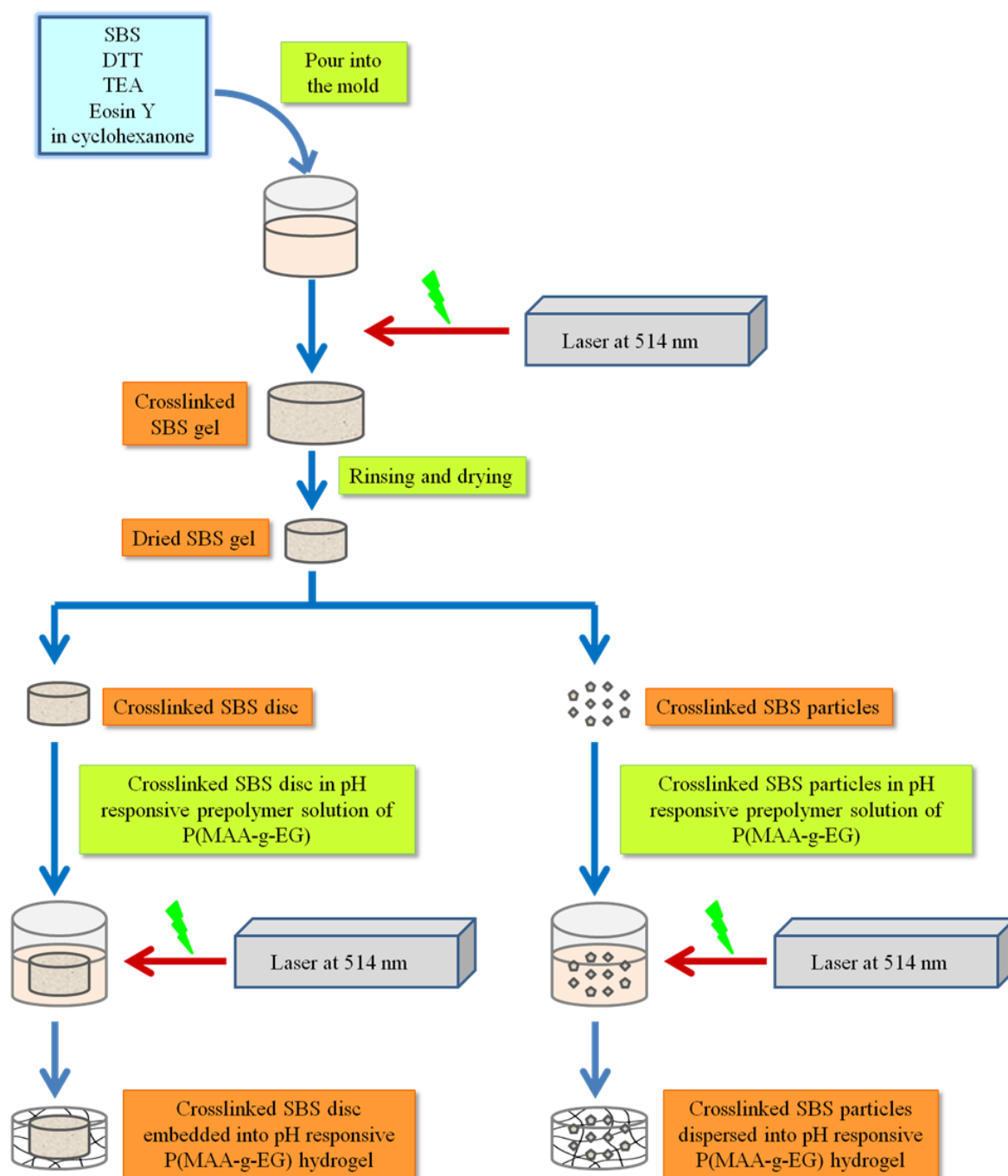


Figure 3.1 Scheme for synthesis of hydrophobic SBS formation and its subsequent encapsulation within pH responsive hydrogels.

3.2.2 Characterization

3.2.2.1 Water Contact Angle Measurement (WCA)

Wettability of the sample is directly related with the surface energy of the hydrogel. However, there is no straight way to measure the surface tension or surface energy of the sample. On the other hand, there are some indirect measurement methods, which are depended on water contact angle measurements [84]. Thus, hydrophobicity of crosslinked SBS gels was characterized by water contact angle measurement (WCA).

In order to calculate surface tension of the solids, there is a common relation at three-phase boundary which is expressed as follows [84]:

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} * \cos\theta \quad (1)$$

where γ_{sv} is solid-vapor surface tension, γ_{sl} is solid-liquid surface tension, and γ_{lv} is liquid-vapor surface tension [Figure 3.2].

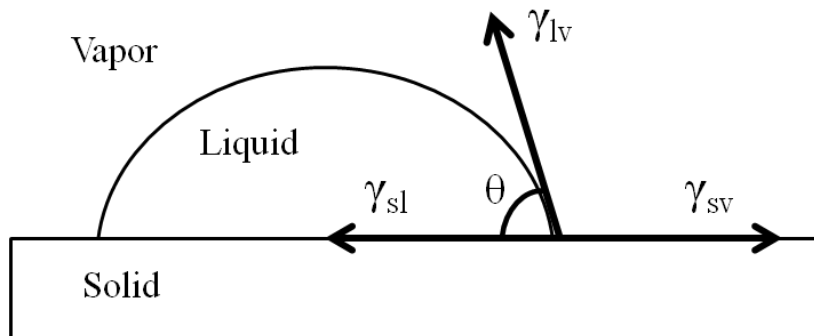


Figure 3.2 Equilibrium contact angle.

WCA measurements were performed using Krüss G10 goniometer. WCA on crosslinked SBS surfaces were measured at room temperature and ambient relative humidity using the goniometer. Ten μl drop of distilled water was placed on SBS gel surface and water contact angle was measured. Contact angle of crosslinked SBS is measured for at least three samples with a standard deviation of $\pm 5^\circ$.

3.2.2.2 Scanning Electron Microscopy (SEM)

Scanning electron microscope (SEM) images were used to investigate the final microstructure and uniformity of pore size for the gels that were synthesized with UV or visible light. For SEM imaging, all samples were dried under vacuum and fixed on carbon tape attached specimen stubs. A thin layer of gold (3.5 nm thickness) was coated on samples using a Cressington Sputter Coater (108Auto with Cressington Thickness Monitor MTM-10). All samples were observed using the Zeiss Evo LS15 scanning electron microscope at an acceleration potential of 5 kV. Next, the images were taken using automatic image capture software.

3.2.3 Swelling Experiments

3.2.3.1 Dynamic Swelling Experiments of P(MAA-g-EG) and Hybrid Hydrogels

To analyze swelling behavior and characterize pH sensitivity of hybrid hydrogels, dynamic weight swelling experiments were carried out in various pH buffer solutions. 0.1 M DMGA buffer solutions were prepared as in the range of pH: 3.8 to pH: 7.0. The hydrogels were kept in DMGA buffer solution with a specific pH at 37 °C incubator, and weighed at specified time intervals for the calculation of equilibrium weight

swelling ratio. The weight swelling ratio, q represents the swelling ratio of the network and was calculated using the following equation:

$$q = W_s / W_d \quad (2)$$

where W_s is the weight of the swollen hydrogel and W_d is the initial weight of the dried hydrogel.

3.2.3.2 Reversible Swelling Experiments of P(MAA-g-EG) and Hybrid Hydrogels

In addition to dynamic swelling experiment, reversible swelling/deswelling behavior of hydrogels was characterized using 0.1 M DMGA buffer solutions with pH values within 2.2-7.0 range at 37 °C. The main purpose of using this pH range is, to mimic the drug reversible swelling profile in both stomach and intestine. Thus, we carried out these experiments at low pH (2.2) and high pH (7.4) buffers as in the literature [85, 86].

For all these experiments, ionic strength of DMGA buffers were maintained with sodium chloride and the temperature of the medium were kept stable at 37 °C incubator.

3.2.4 Pregabalin (PGB) Loading

PGB loading studies were completed with visible light cured P(MAA-g-EG) hydrogels and hybrid hydrogels. PGB stock solution was prepared in 1 x PBS (pH: 7.4) at a concentration of 0.25 mg/ml. pH sensitive P(MAA-g-EG) hydrogels and composite hydrogels were incubated for 16 hours in 10 ml PGB stock solution. Next, hydrogels were collapsed with the addition of 2 μ l of 6 M HCl in order to retain the drug within

the network. Finally, to eliminate drug adsorption on the surface, PGB loaded hydrogels were rinsed with distilled water.

UV-visible spectrophotometer (Shimadzu UV-3600 – UV-VIS-NIR Spectrophotometer) at 210 nm wavelength was used to measure PGB absorption. Standard calibration curve for the absorption of PGB in aqueous solution was generated for determination of concentration in unknown samples. Loading efficiency of PGB into hybrid gels was calculated as follows [59]:

$$\text{Loading efficiency} = (M_0 - M_f) / M_f * 100 \quad (3)$$

where M_0 represents PGB mass in the stock solution initially and M_f is the final PGB mass remaining in the solution at the end of 16 hours.

3.2.5 PGB Release Studies

For PGB release studies, PGB loaded hydrogels were placed in 1 x PBS (pH: 7.4) at 100 rpm and 37°C. For every 30 minutes up to 7 hours, 500 µl samples were taken and replaced with 1 x PBS (pH: 7.4) to sustain sink conditions. On the other hand, the same experiment was repeated at low pH conditions. For this case, solution buffer was replaced with 1 x PBS at a pH of 2. PGB concentrations obtained from absorption measurements were used to calculate mass released at time t (M_t) using the following equation [87]:

$$M_t = C_t * V + \sum C_{t-1} * V_s \quad (4)$$

where C_t is the concentration of PGB in the release solution at time t , V is the total volume of release solution (10 ml) and V_s is the sample volume that is taken (500 μ l). Using the M_t values, the % release of PGB was determined as expressed with the following equation [59]:

$$\% \text{ Mass Released} = (M_t/M_\infty) * 100 \quad (5)$$

where M_∞ is the total weight of PGB released during the experiment. Once the diffusion behavior of PGB from pH responsive and hybrid hydrogels were obtained, the results were modeled using the power-law correlation developed by Peppas *et al.* [8] as follows:

$$M_t/M_\infty = kt^n \quad (6)$$

where k represents the characteristic constant of the hydrogel and n is the exponential coefficient, which describes the mode of transport mechanism. The value of n has been reported as 0.45 for Fickian Diffusion and 0.89 for swelling-controlled diffusion for cylindrical geometry [5]. The fittings were done based on the first 60% of the collected release data to obtain a more accurate fit.

3.2.6 Cytocompatibility of pH Responsive Hydrogels

3.2.6.1 *In vitro* Cell Viability Tests

To analyze *in vitro* cell viability of P(MAA-g-EG) and hybrid hydrogels, human fibroblast cell line (Passage number: 8-10) was used. Cells were cultured at 37°C with 5% CO₂ in an incubator and maintained in DMEM (Paisley, UK) which was supplemented with 1% Penicillin/Streptomycin, 2 mM L-Glutamin and 10% FBS. The

P(MAA-g-EG) and hybrid hydrogels were immersed in the complete culture medium for 24 h, at 37°C before seeding. For static seeding of the fibroblast cells, all hydrogel samples were located in 24-well plate. Then, 50 µl fibroblast cell suspensions (3×10^5 cell/mL) were seeded onto the hydrogels. After 15 minutes, 1 mL of complete culture medium was added to each well and the hydrogels were placed in the incubator for 24 h. In order to characterize the metabolic activity of fibroblast cells on hydrogels, CellTiter-Glo luminescent cell viability assay (Promega) was used on day 1 and day 4. Standard curve was generated using ATP solutions at specific concentrations (1, 0.1 and 0.01 µM) prepared in cell culture medium. Next, 150 µl from each hydrogel medium was taken and placed in the same amount of CellTiter-Glo Reagent. Both ATP solutions and samples were incubated at 25°C for 15 minutes and then, luminescence was measured using a plate reader (Biotek, Synergy H1).

3.2.6.2 *In vivo* Implantation Experiments for Irritation or Intracutaneous Reactivity Test

According to the ISO Standard, irritation means that a localized inflammatory response to a single or continuous application of the test sample. That's why, here the irritation test was used to evaluate the irritation potential of pH sensitive P(MAA-g-EG) hydrogels. With this purpose, the irritation tests were carried out under standardized conditions of ISO 10993-10, ISO 10993-2 and ISO 10993-12 [88-90]. Three healthy female New Zealand rabbits were used to investigate irritation or intracutaneous reactivity of the hydrogels. Fluid extracts from 0.2 g/mL (w/v) of the dried pH

responsive hydrogel was directly applied on the right and left back skin of the rabbits. After that, rabbits were monitored for 72 h.

The left caudal zone of the back was used as positive control (90% lactic acid) whereas the right side was used as a negative control (physiological saline solution) as shown in Figure 3.3.

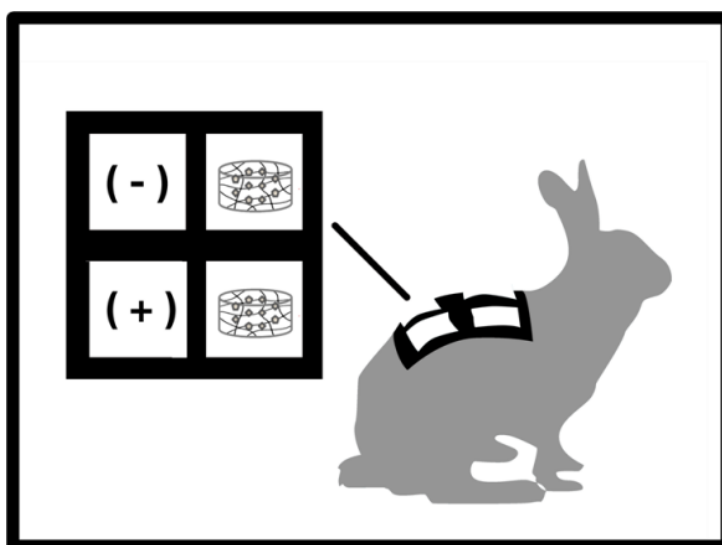


Figure 3.3 Representative image of the application sites of the rabbit.

At 1, 24, 48, and 72 hours after injection, the intracutaneous reaction was assessed using the evaluation criteria. The criteria of classification of the intracutaneous implantation sites are shown in Figure 3.3.

Table 3-1 Evaluation criteria of the intracutaneous reactions.

Average Score	Erythema & Edema
0 – 0.4	Insignificant
0.5 – 1.9	Slight
2 – 4.9	Moderate
5 - 8	Severe

3.2.6.3 Histological Analysis

To investigate the histological analysis, three types of samples were taken from the negative control sites, positive control sites and test sites of the rabbits. Then, these tissue samples were washed and dehydrated respectively. After that, tissue samples were prepared with a 5 μ m thickness and “hematoxylin and eosin staining assays” were carried out. Next, the tissues were analyzed under microscope.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization

Composite hydrogels that include crosslinked SBS were synthesized with both UV and visible light initiated photopolymerization. Prepolymer solutions of pH responsive monomers included Irgacure and eosin/TEA photoinitiation system for UV and visible light induced photopolymerization, respectively. First, crosslinked SBS gels were prepared, which were cured under visible light, then crosslinked SBS in the form of disc or particles were added into prepolymer solutions of P(MAA-g-EG) hydrogel. Next, photopolymerization of hybrid P(MAA-g-EG) hydrogel networks was carried out via both UV light and visible light exposure. Surface hydrophobicity of crosslinked SBS was characterized by water contact angle measurement (WCA) and surface porosity of SBS gels and hybrid hydrogels were analyzed using scanning electron microscope (SEM).

In addition to these characterization methods, visual examination of the three types of hydrogels shown in Figure 4.1. P(MAA-g-EG) hydrogel, P(MAA-g-EG) hydrogel that included SBS disc and P(MAA-g-EG) hydrogel that included SBS particles, demonstrated opaque appearance at low pH (pH: 2.2), while they appeared soft and transparent when the pH was 7. As shown in Figure 4.1, crosslinked SBS disc was

remained in the middle section of the P(MAA-g-EG) hydrogel, whereas SBS particles were distributed throughout the P(MAA-g-EG) hydrogel network. Also, both the composition of hydrogel prepolymer and the photoinitiation mechanism have an influence on the integrity of the gels. For instance, Figure 4.1(b) and Figure 4.1(d) demonstrate that three types of pH sensitive hydrogels formed under visible light, preserved their integrity better than the ones that were formed under UV light when they were under swollen condition.

In addition, Figure 4.1(b) showed that crosslinked SBS particles have a positive influence on the integrity of the hydrogel. This integrity could be due to the homogenous distribution of crosslinked SBS particles throughout the pH responsive solution, which might have promoted the diffusion of eosin from the hydrophobic network towards pH responsive hydrogel, and hence resulted in better integrity compared to other samples.

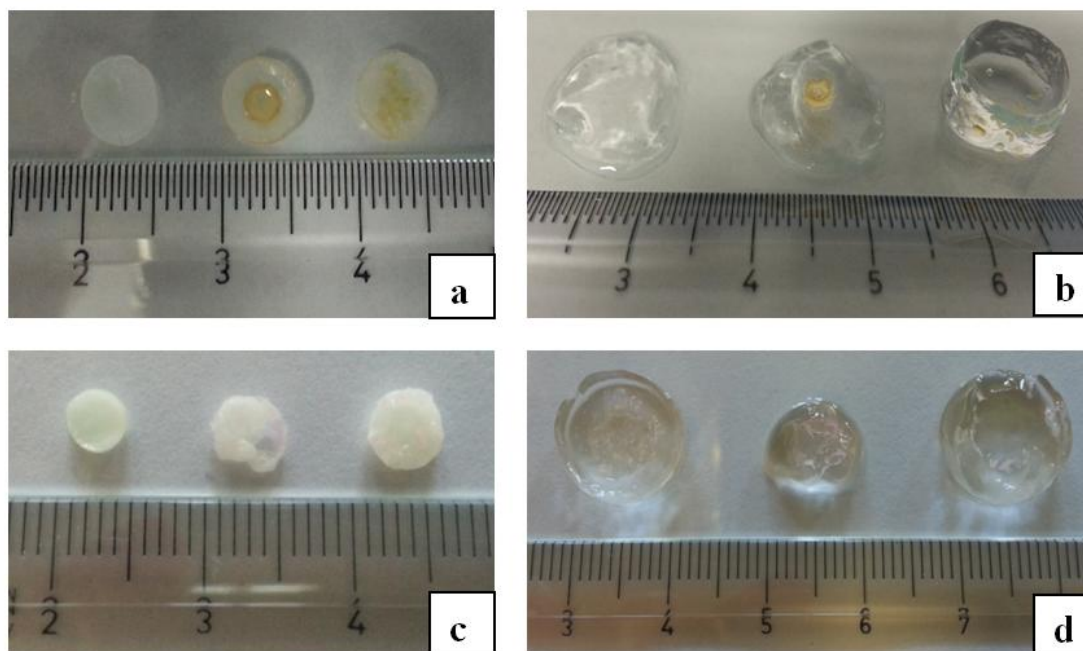


Figure 4.1 Image of pH responsive P(MAA-g-EG) hydrogel, crosslinked SBS disc embedded into P(MAA-g-EG) hydrogel and crosslinked SBS particles dispersed into P(MAA-g-EG) hydrogel, which had been photopolymerized under UV light, and are (a) collapsed at pH: 2.2, or (b) swollen at pH: 7.0 DMGA buffer solutions. Image of the same three different types of hydrogels, which had been prepared with eosin Y and TEA, and photopolymerized under visible light, and are (c) collapsed at pH: 2.2, or (d) swollen at pH: 7.0 DMGA buffer solutions.

4.1.1 Characterization of SBS Gels

Hydrophobicity of crosslinked SBS gel was characterized by WCA and Figure 4.2 demonstrated that the contact angle of water droplet for crosslinked SBS surface was $64.5^{\circ} \pm 5$. The hydrophobicity of the crosslinked SBS surface was less hydrophobic than thin film surfaces prepared with linear SBS [91]. Moreover, WCA measured for crosslinked SBS is different from contact angle measurements reported by Kennedy and

Higginbotham, where 113° as contact angle was measured on SBS polymer [67]. The decreases observed in water contact angle could be because of the presence of hydrophilic crosslinker DTT. Use of DTT during crosslinking reaction could cause a decrease in the surface hydrophobicity. That's why, the hydrophobicity of SBS gel can be controlled by changing of the crosslinker amount in the prepolymer solution. As an alternative approach, higher hydrophobicities could be achieved by using conjugation of functional groups, such as methyl, into the SBS structure [65].

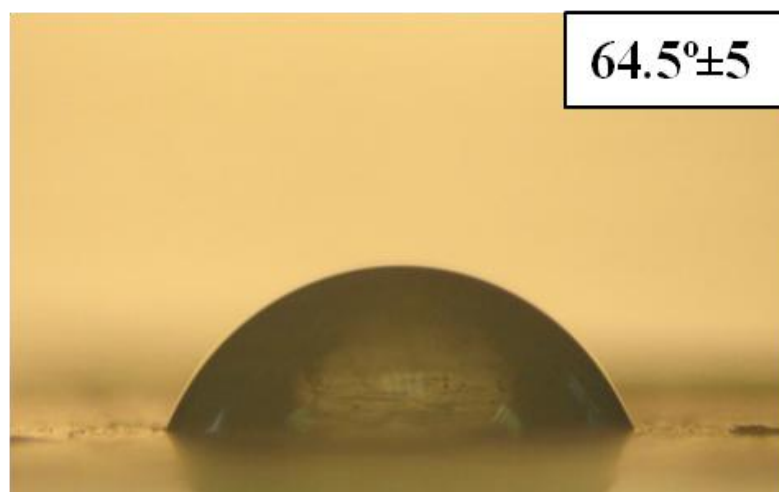


Figure 4.2 Image of a water droplet on crosslinked SBS gel.

As another characterization method, SEM was used to analyze and compare the surface of SBS polymer and crosslinked SBS gel. Figure 4.3(a) showed that a film of the linear SBS polymer was very stiff network without pores. Also, this result is consistent with the previous literature study which had been published by Kennedy *et al.* for linear SBS polymer films [67].

Besides, crosslinked SBS gels were obtained by photopolymerization reaction of SBS polymer with eosin Y, TEA and DTT. The crosslinked SBS gel had a porous structure as shown in Figure 4.3(b) and the average pore size was 316 ± 160 nm.

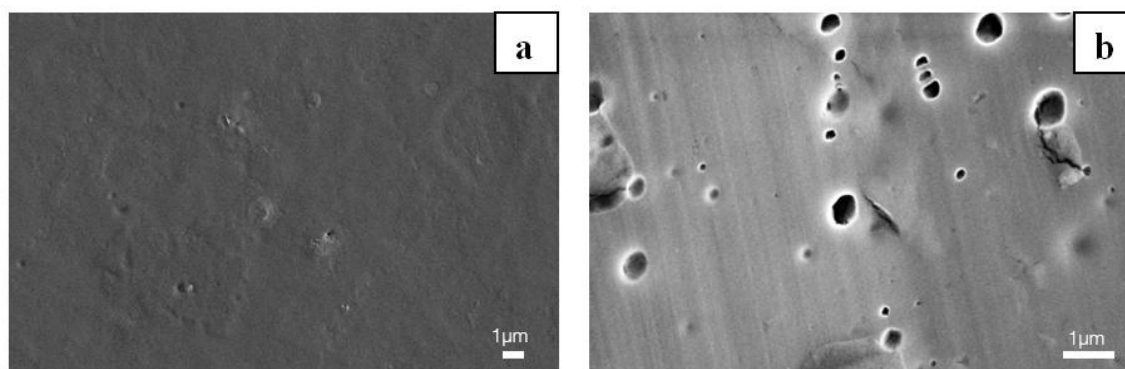


Figure 4.3 SEM image of (a) SBS polymer and, (b) crosslinked SBS gel that was photopolymerized under visible light.

4.1.2 Characterization of Hybrid Hydrogels

SEM images demonstrated that visible light induced photopolymerization had an effect on microstructure of hydrogels. Also this method, resulted in the formation of uniform structure and larger pores on the surface when compared to the condition of UV light exposure [Figure 4.4].

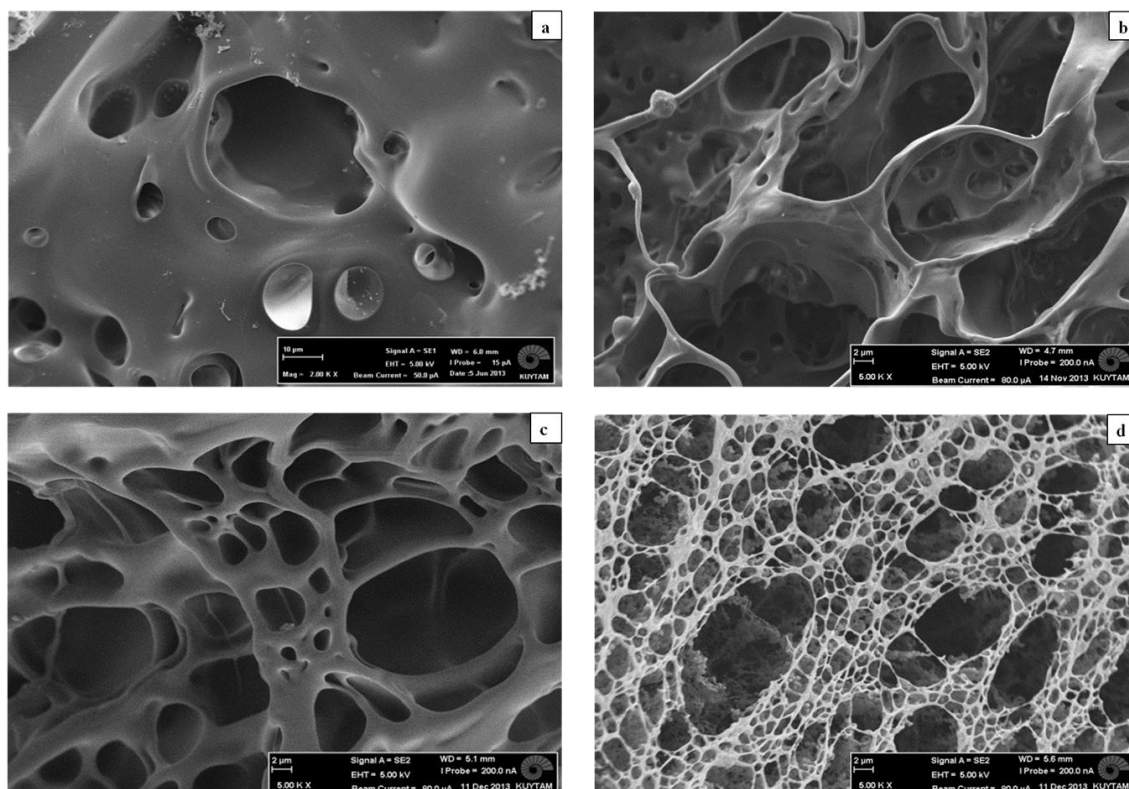


Figure 4.4 Scanning electron microscope images of (a) pH responsive hydrogel P(MAA-g-EG) photopolymerized under UV light, (b) pH responsive hydrogel P(MAA-g-EG) photopolymerized under visible light, (c) pH responsive hydrogel P(MAA-g-EG) that includes crosslinked SBS disc photopolymerized under visible light, (d) pH responsive hydrogel P(MAA-g-EG) that includes crosslinked SBS particles photopolymerized under visible light. (Scale bar: 2 μm).

A pore size range between 0.6 – 22.4 μm was obtained with visible light exposure, whereas pore diameter changed between 2.2 – 37.3 μm with UV light exposure. Thus, visible light cured hybrid hydrogels have uniform pore microstructure depending on the photoinitiation mechanism. Despite higher porosities and large pore sizes, better integrities were observed for hydrogels synthesized with visible light, compared to the

ones synthesized with UV light exposure Figure 4.4(a,b). This could be due to the efficient radical formation and crosslinking reactions taking place with visible light exposure in the presence of eosin Y and TEA, and hence this results in better integrity.

On the other hand, with addition of crosslinked SBS in the form of disc or particles into the P(MAA-g-EG) prepolymer solution, both stiffer structures and smaller pore diameters were obtained as shown in Figure 4.4(c,d). The pore size of P(MAA-g-EG) hybrid hydrogel, which consists of SBS disc, ranged between 1.5 – 14.2 μm . Besides, P(MAA-g-EG) hydrogel with crosslinked SBS particles, showed further decreases in pore diameter with a range of 0.2 – 18.4 μm . This reduction in pore size with the addition of SBS could be because of diffusion of excess eosin Y from crosslinked SBS. That's why, eosin Y could allow to synthesize higher crosslinked P(MAA-g-EG) hybrid hydrogels with smaller pore sizes. This result also supports previous studies of Kizilel *et al.* with poly(ethylene glycol) diacrylate (PEGDA) hydrogels, where eosin Y have been found to increase crosslinking reactions and lead to higher crosslink densities [61, 92-94]. Furthermore, better diffusion of eosin Y occurs in the case of homogenous distribution of crosslinked SBS particles throughout the prepolymer. As a result, tighter network was formed when compared to the P(MAA-g-EG) hydrogel having SBS disc in the middle [Figure 4.4(c,d)].

4.2 Swelling Experiments

4.2.1 Dynamic Swelling Behavior of P(MAA-g-EG) and Hybrid Hydrogels

Dynamic swelling experiments were performed to investigate the pH sensitive behavior of the hybrid hydrogels [Figure 4.5]. Thus, simulated physiological buffer solution with different pH values were used in swelling studies. Also, the results of dynamic swelling experiments demonstrated the effect of both addition of the crosslinked SBS and wavelength of the light used for photopolymerization reaction.

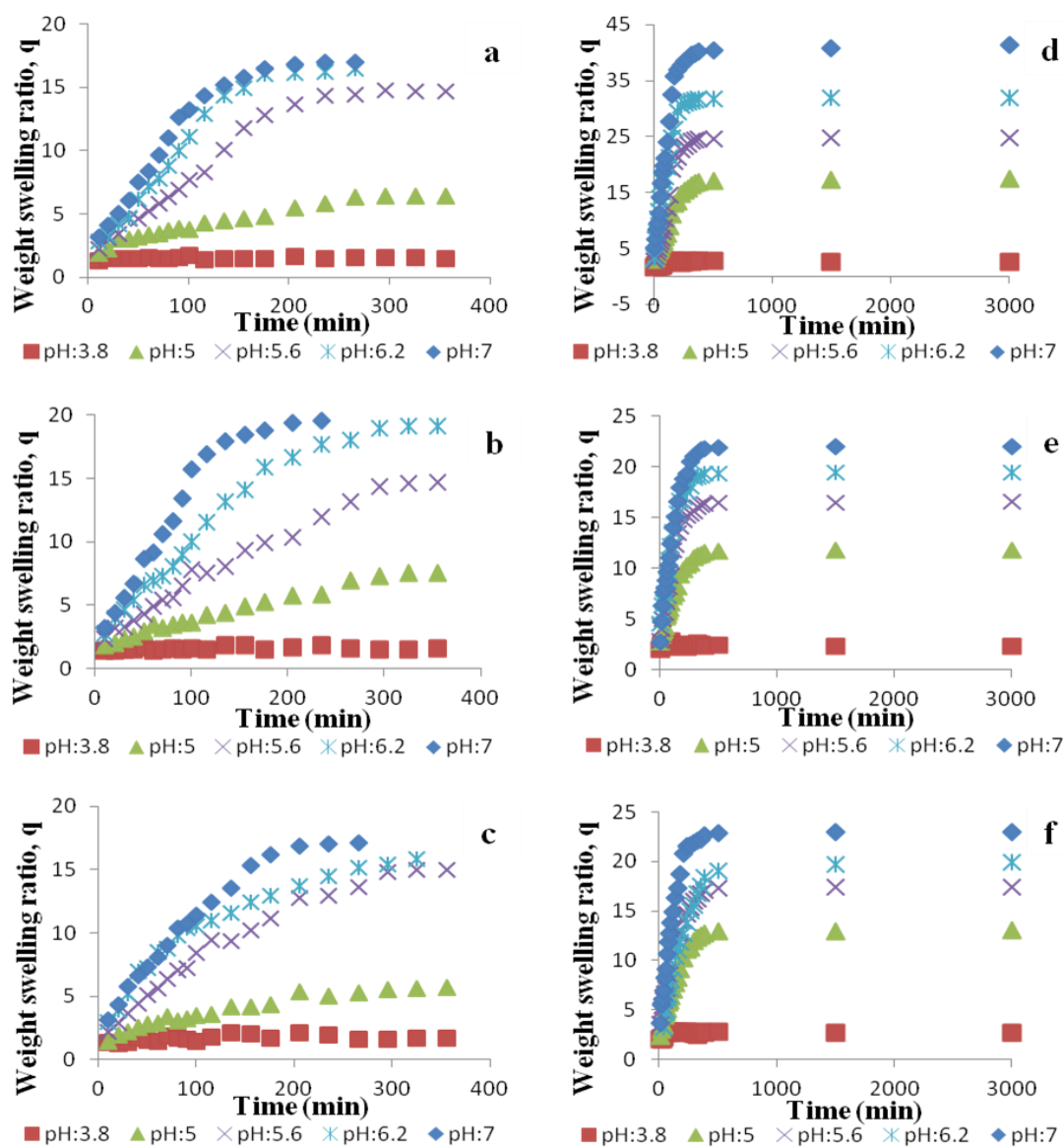


Figure 4.5 Dynamic swelling behavior of (a) pH responsive hydrogel P(MAA-g-EG) (b) crosslinked SBS disc embedded into P(MAA-g-EG) hydrogel, (c) crosslinked SBS particles dispersed into P(MAA-g-EG) hydrogel that were photopolymerized under UV light. Dynamic swelling/deswelling behavior of (d) pH responsive hydrogel P(MAA-g-EG) (e) crosslinked SBS disc embedded into P(MAA-g-EG) hydrogel, (f) crosslinked SBS particles dispersed into P(MAA-g-EG) hydrogel that were photopolymerized under visible light.

At high pH value, the carboxylic groups of MAA become ionized, hence electrostatic repulsion occurs between neighboring PEG chains. PEG chains take part in hydrogen bonding with water, and also supply significant coil extension. On the other hand, at low pH condition, the carboxylic groups of MAA monomer become protonated. As a result, hydrogen bonding between MAA and PEG chains is generated. That's why collapsed state is obtained at low pH conditions for pH responsive hydrogels [17, 95]. As shown in Figure 4.5, we observed minimal swelling in acidic condition, while maximum swelling in basic environment and this situation was also reported in other studies [96]. When longer durations were applied for swelling experiments, visible light cured hybrid hydrogels showed higher swelling ratios than UV photopolymerized ones [Figure 4.5]. Swelling ratios of P(MAA-g-EG) hydrogels synthesized with visible light were higher than the hydrogels which formed with UV light. The results of the swelling ratio of UV photopolymerized hydrogels were also parallel to the previous literature studies that used similar conditions in their experiments [96].

In addition, Figure 4.5 shows that the analysing of long term swelling behavior which was obtained for pH sensitive hybrid hydrogels. According to these swelling experiments, addition of crosslinked SBS into the pH responsive hydrogels that were photopolymerized with UV light do not cause significant changes in swelling [Figure 4.5(a-c)]. On the other hand, this examination was not valid for visible light induced pH responsive hybrid hydrogels. Thus, the incorporation of crosslinked SBS in the form of

disc or particles into the P(MAA-g-EG) hydrogels decreased swelling as shown in Figure 4.5(d-f).

As a separate note, these swelling results could be explained by the number of pores which were different in hybrid hydrogels based on their photopolymerization mechanisms. Although visible light cured hydrogels had smaller pore sizes, their swelling ratios were higher than UV polymerized ones. This could be because of the higher number of pores that are present in hydrogels synthesized with visible light, since higher number of pores lead to improved swelling ratios. Moreover, this result was also supported by SEM images as in Figure 4.4 where UV polymerized hydrogel had fewer number of pores compared to the hydrogel synthesized with visible light.

In previous studies, critical parameters of hydrogels have been calculated by using swelling ratios of the hydrogels. Critical conditions are related to the degree of ionization which is the dominant factor for ionic networks. Furthermore critical conditions would require the parameters such as X_1 , X_2 , to find the regime that brings the system close to the critical state [97].

4.2.2 Reversible Swelling Behavior of P(MAA-g-EG) and Hybrid Hydrogels

Reversibility of swelling behavior was also observed for composite hydrogels synthesized both UV and visible light. For these tests, hydrogels were incubated at low pH (pH: 2.2) and high (pH: 7.0) respectively in DMGA buffer solutions.

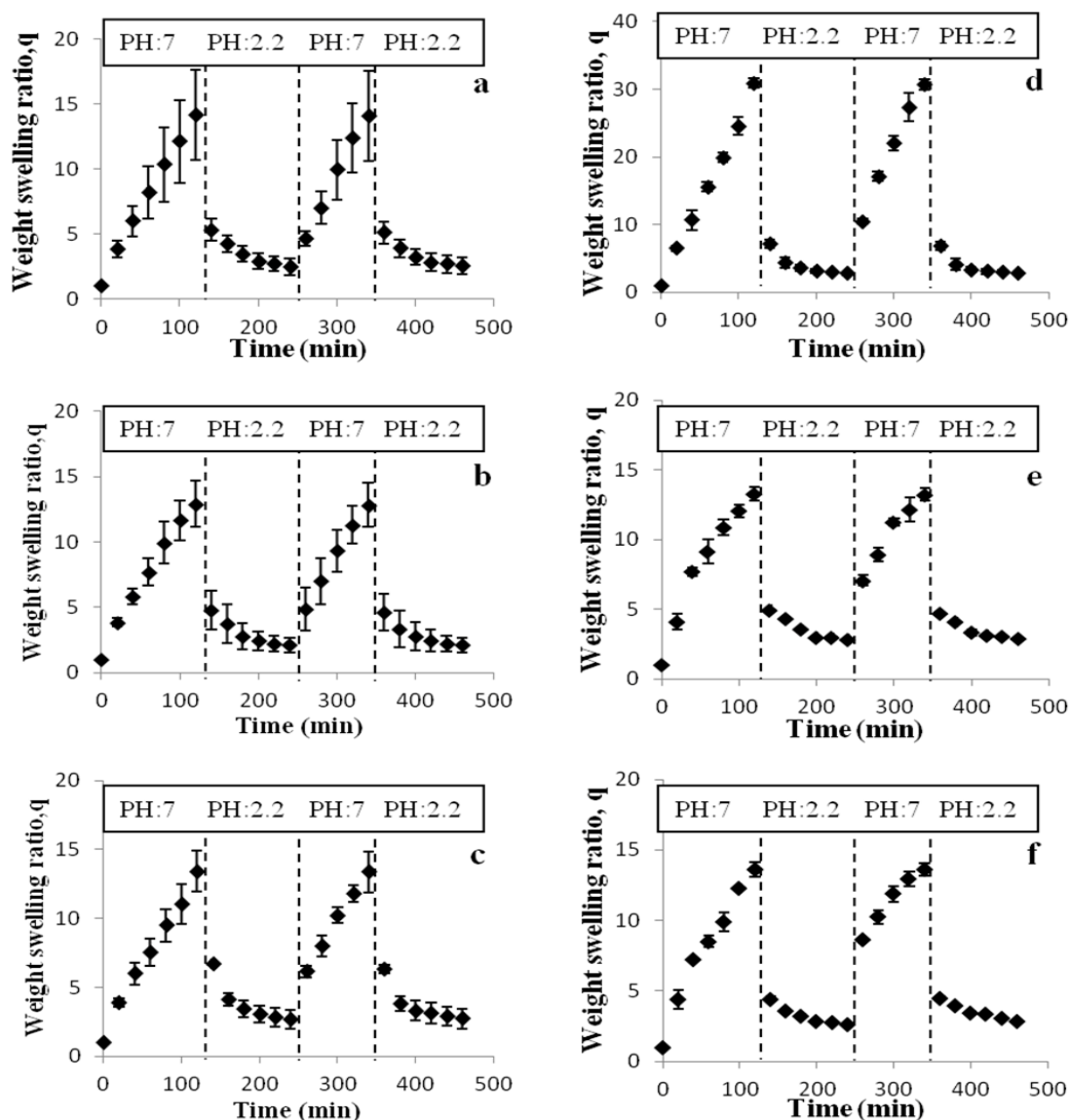


Figure 4.6 Reversible swelling/deswelling behavior of (a) pH responsive P(MAA-g-EG) (b) crosslinked SBS disc embedded into P(MAA-g-EG), (c) crosslinked SBS particles dispersed into P(MAA-g-EG) hydrogel which all had been photopolymerized under UV light. Reversible swelling/deswelling behavior of (d) pH responsive P(MAA-g-EG) (e) crosslinked SBS disc embedded into P(MAA-g-EG), (f) crosslinked SBS particles dispersed into P(MAA-g-EG) hydrogel which all had been photopolymerized under visible light.

Figure 4.6 demonstrates that, hydrogels polymerized under different conditions can respond to changes in pH values. With repeated changes in pH, P(MAA-g-EG) hydrogels swell and collapse depending on the changing of pH. Also it was observed that, the highest swelling was achieved for P(MAA-g-EG) hydrogel polymerized with visible light.

Incorporation of SBS disc or particles into the P(MAA-g-EG) hydrogels have a significant effect on weight swelling ratios. Lower weight swelling ratios were observed in Figure 4.6(e) and Figure 4.6(f) which was the case of usage of visible light exposure. This situation could be also because of the diffusion of excess eosin from SBS gel to the P(MAA-g-EG) network. That's why, tighter network structure was occurred and lower swelling ratios were obtained [Figure 4.6(d-f)].

4.3 Pregabalin (PGB) Loading

Pregabalin loading and release experiments were completed with only P(MAA-g-EG) hydrogels that synthesized with visible light induced polymerization. Different PGB loading efficiencies were observed, since hybrid hydrogels have various network structure and composition. As shown in Table 4-1, PGB was successfully loaded as $56.7 \pm 8.3\%$, $62.3 \pm 1.7\%$ and $63.5 \pm 2.4\%$ into P(MAA-g-EG), SBS disc incorporated P(MAA-g-EG), and SBS particle incorporated P(MAA-g-EG) hydrogel, respectively.

Table 4-1 Comparison of % loading efficiencies, weight swelling ratio, and % PGB release for pH responsive hydrogels.

Formulation of Hydrogel	% Loading Efficiency	Weight Swelling Ratio	% PGB Release
Pure P(MAA-g-EG)	56.7±8.3	40	86.4
SBS disc embedded into P(MAA-g-EG)	62.3±1.7	22	74.3
SBS particles into P(MAA-g-EG)	63.5±2.4	23	79.5

Loading efficiencies of the drug were slightly increased with addition of SBS into the pH sensitive network as shown in Table 4-1. This could be a result of the presence of SBS in the P(MAA-g-EG) network, since SBS could provide additional sites for loading of the pregabalin. Also, the amount of SBS was kept constant for both types of hybrid hydrogels and similar percent loading results were obtained.

In addition, crosslinked SBS gel size might have also an influence on drug loading capacity. For instance, incorporation of SBS particles with a smaller size might increase the loading of PGB as demonstrated in Table 4-1. Addition of SBS gel into the pH responsive system in the form of particles instead of a single disc resulted in higher amount of PGB release. Also Table 4-1 shows that, the highest amount of PGB release was observed with the highest swelling ratio, which was for the case of pure pH responsive P(MAA-g-EG) hydrogels.

This is a visible-light-induced hydrogel network so unreacted double bonds may further crosslink when exposed to light for extended durations. This was not investigated in the current work and will be considered in our future studies.

4.4 PGB Release Studies

Pregabalin release experiments were performed at constant pH buffer solution where drug release profiles were observed for different hydrogel samples [98]. Figure 4.7 demonstrates that in neutral pH buffer solution (pH: 7.4), 86.4% of PGB was released from pH sensitive P(MAA-g-EG) hydrogel within 400 minutes. On the other hand, 15.3% of PGB release was occurred in a acidic buffer solution with pH: 2.2.

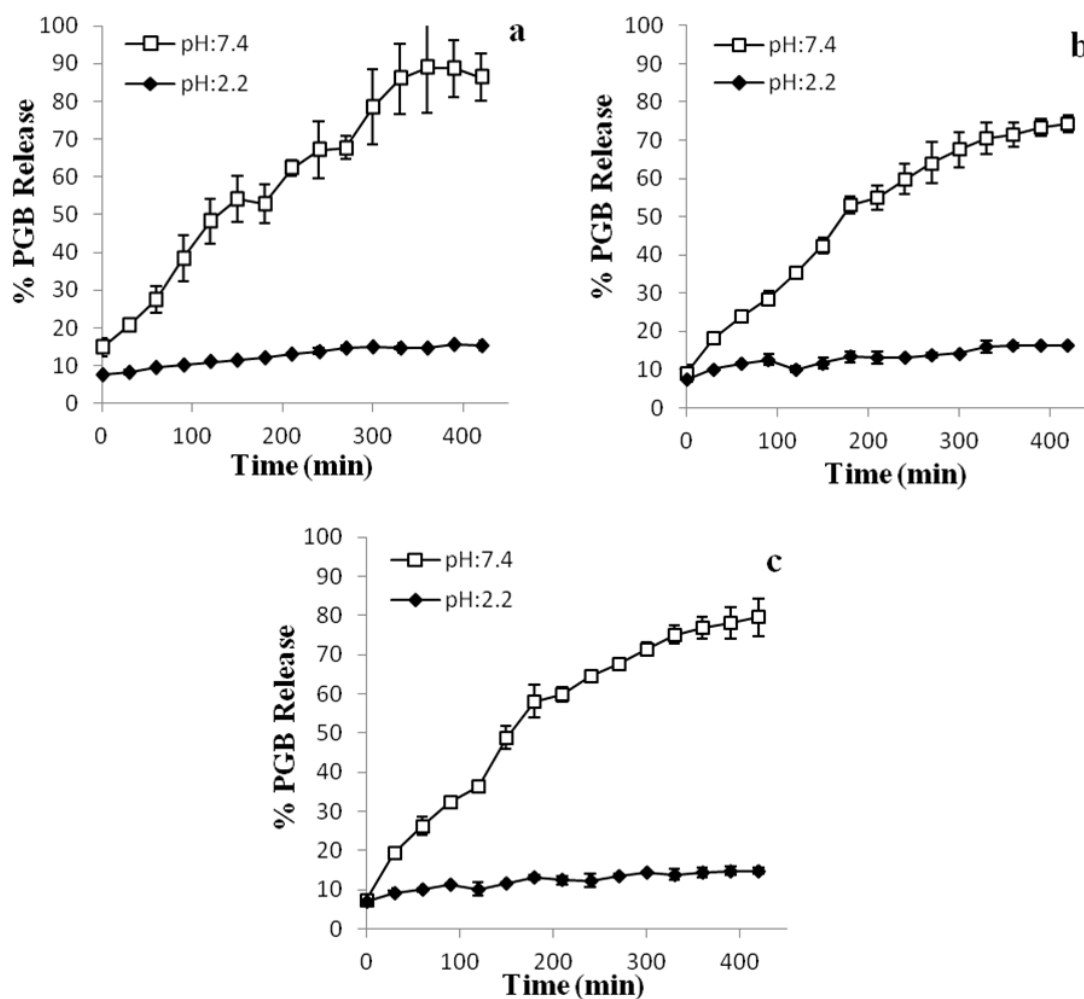


Figure 4.7 Release behavior of PGB at pH: 2.2 and pH: 7.4 from (a) P(MAA-g-EG) hydrogel , (b) SBS embedded into P(MAA-g-EG) hydrogel, and (c) SBS particles dispersed into P(MAA-g-EG) hydrogel that were photopolymerized under visible light.

Above the pK_a value of the MAA, which is 5.4 [99], pH sensitive hydrogel maintained the swollen state and provided higher drug release at pH: 7.4 medium. In addition, Figure 4.7 shows that PGB release from hybrid P(MAA-g-EG) hydrogels were decreased when compared to pure pH sensitive P(MAA-g-EG) hydrogel.

In the case of hybrid hydrogels with crosslinked SBS disc, 74.3% PGB release was observed at high pH medium whereas, at low pH only 16.4% PGB was released [Figure 4.7(b)]. However, SBS particles incorporated composite hydrogels provided that 79.5% and 14.9% of the PGB was released at pH: 7.4 and at pH: 2.2 respectively [Figure 4.7(c)]. These results showed that at low pH environment, minimum amount of PGB release was observed for all types of hydrogels, as expected. Also, this judgment is consistent with the previous literature study which is about the delivery of various types of drugs from pH sensitive networks at acidic pH [100]. For instance, Barnerjee *et al.* investigated the release of a non-steroidal anti-inflammatory drug from pH responsive network depending on the pH changes from 2.2 to 7.4 [101].

The transport of PGB from each hydrogel sample (pure pH responsive, SBS disc embedded and SBS particle containing) was modeled using equation (6). Exponent n in the equation describes the mode of transport, and was determined for each condition at high (pH: 7.4) and low (pH: 2.2) pH conditions. Values of n closer to 0.45 designate a Fickian mode of diffusion, whereas values closer to 0.89 designate a swelling-controlled diffusion behavior. Swelling controlled release was also reported by Peppas *et. al.* [5] where n values higher than 0.89 was found [96]. Here, we also observed that at pH: 7.4 the value of n was higher than 0.89 for pH responsive P(MMA-EG). It was observed that the values of n were lower for low pH conditions for all types of gels Table 4-2. This is expected as the pH sensitive P(MAA-g-EG) collapses at this condition. Moreover, SBS-containing gels exhibited lower n values meaning Fickian

diffusion forces were more dominant both at lower and higher pH values compared to the diffusion through pure P(MAA-g-EG) hydrogel.

Table 4-2 Power law coefficient (n) values for pH responsive hydrogel and its composite forms with crosslinked SBS at pH: 2.2 and pH: 7.4.

Formulation of Hydrogel	n at pH:2.2	n at pH:7.4
Pure P(MAA-g-EG)	0.0897 ± 0.076	1.054 ± 0.102
SBS disc embedded into P(MAA-g-EG)	0.387 ± 0.259	0.806 ± 0.096
SBS particles into P(MAA-g-EG)	0.580 ± 0.126	0.800 ± 0.092

In addition, when crosslinked SBS gel added into hydrophilic pH responsive P(MAA-g-EG) network, percent PGB release was decreased. This is because of the increment of hydrophobicity of P(MAA-g-EG) hydrogels with addition of SBS disc or particles. Thus, PGB release from composite hydrogels was decreased as shown in Figure 4.7. The crosslinked SBS domains of the composite pH responsive hydrogels could have reduced the release of hydrophilic PGB and as a result, PGB delivery from the network is diminished [59]. Furthermore, composite hydrogels having SBS particles displayed higher amount of PGB release than the hybrid hydrogels that containing SBS disc as appears in Figure 4.7(b,c). This is based on the type of the hybrid hydrogel, since regular distribution of SBS particles within the P(MAA-g-EG) network or embedding of SBS disc in the middle of the hydrogel have an effect on the PGB release.

Additionally, the higher release of PGB from pure pH sensitive P(MAA-g-EG) hydrogels [Figure 4.7(a)] can be clarified with greater swelling response in the case of pH changes [Figure 4.6(d)].

4.5 Cytocompatibility of pH Responsive Hydrogels

4.5.1 *In vitro* Cell Viability Results

For the analysis of cell viability of the pH sensitive hybrid hydrogels, human fibroblast cells were used. That's why, human fibroblasts were seeded into the all types of pH sensitive hydrogels and viability of fibroblasts was observed after 1 and 4 days of incubation. Also, the results of *in vitro* cell viability assay are shown in Figure 4.8.

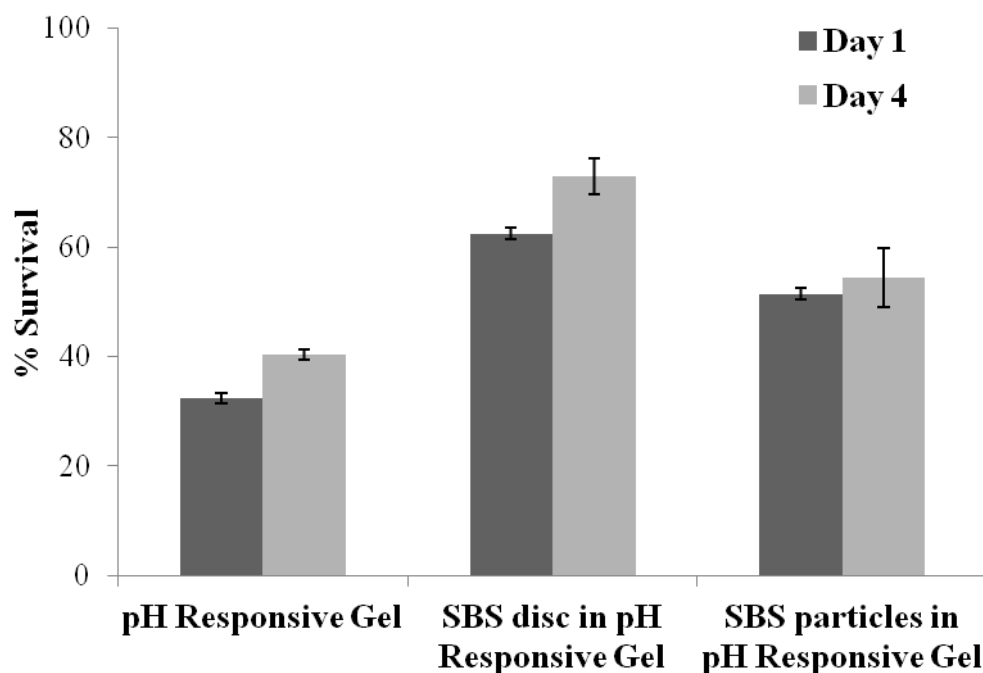


Figure 4.8 Percent survival of human fibroblasts in altered sets of pH responsive P(MAA-g-EG) hydrogels.

Depending on the ATP amounts, percent fibroblast survival was calculated and this result indicates that all types of hybrid hydrogels have no toxic effects on fibroblast growth. Also, pH sensitive hybrid hydrogel having SBS particles exhibited better fibroblast viability as shown in Figure 4.8. This situation can be supported with the stiffness and integrity of the hybrid hydrogel which probably occurs due to the presence of crosslinked SBS. In addition, this could also be demonstrated with better adhesion of fibroblasts on SBS domains, which probably improved focal adhesions and enhanced cell viability.

4.5.2 *In vivo* Implantation Experiments for Irritation or Intracutaneous Recativity Results

In order to investigate any intracutaneous reaction which originate from the hydrogel nature, the irritation assay was performed on rabbits [Figure 4.9(a)]. The results of the assay were compared to the negative control at all observation times of 1, 24, 48, and 72 h. According to the irritation assay, pH responsive P(MAA-g-EG) hydrogel having SBS particles, did not show any intracutaneous reaction such as erythema and edema in rabbits as shown in Figure 4.9(b). Also the images that were taken from the rabbits before in vivo implantation experiments, 4 hours later, and 72 hours later were shown in Figure 4.9(c). Thus, all these observations are significant indications for the biocompatibility of pH sensitive hydrogels that photopolymerized under visible light.

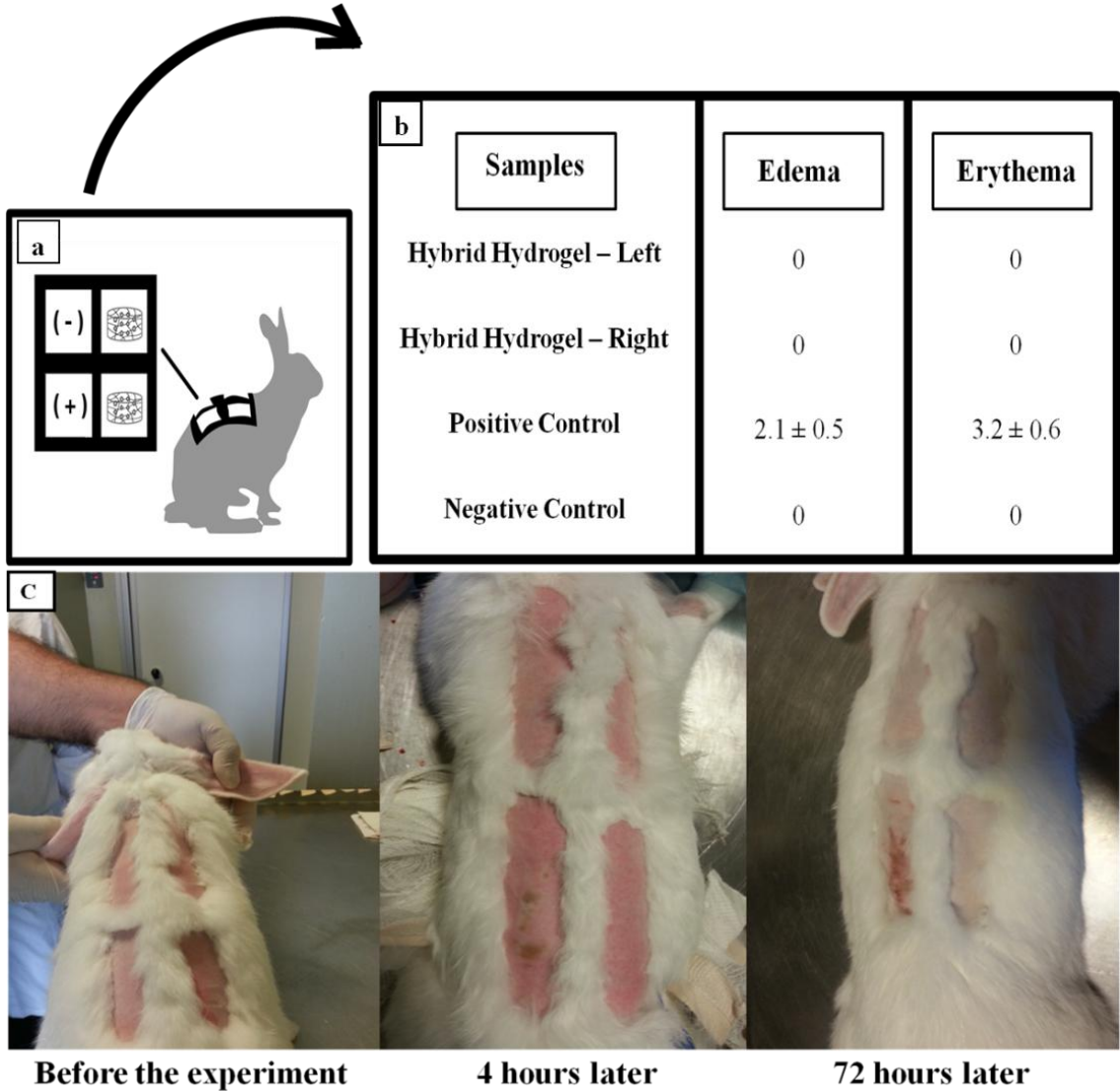


Figure 4.9 *In vivo* implantation experiment results for irritation assay. (a) Schematic representation of or intracutaneous reactivity assay sites, (b) results of intracutaneous reaction. (c) photographic images of implantation experiments; before the experiment, 4 hours later, and 72 hours later.

4.5.3 Histological Analysis

Histological analysis demonstrated that both necrosis in epidermis and heterophilic inflammation in dermis at positive control site, which was consistent with acute dermal irritation. On the other hand, histopathological analysis showed normal morphology at the epidermis and dermis of the both test and negative control sites [Figure 4.10]. Thus the histological analysis further demonstrated that, pH responsive P(MAA-g-EG) hydrogel with SBS particles, did not retain irritative properties.

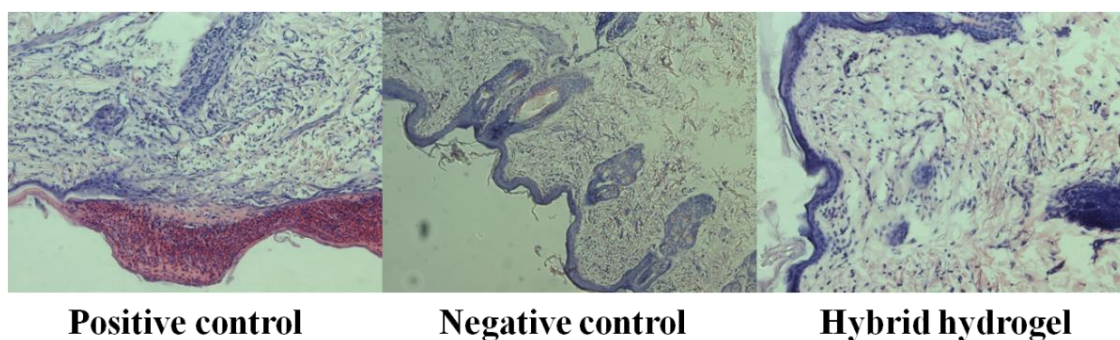


Figure 4.10 Histological images of skin tissue samples from positive control, negative control, and hybrid hydrogel in vivo implantation experiments. All three images were obtained with 20X magnification.

CHAPTER 5

CONCLUSIONS

In biomedical applications, P(MAA-g-EG) hydrogels synthesized under UV light are significant; however, biocompatible and stimuli sensitive hydrogels could also be obtained through visible-light-induced polymerization. In this study, visible light induced synthesis of pH sensitive P(MAA-g-EG) hybrid hydrogels with crosslinked SBS disc or particles were developed for controlled release of pregabalin. First, pure pH responsive P(MAA-g-EG) hydrogel and hybrid hydrogels were photopolymerized under both UV light and visible light. Next, comparison of these hydrogels was carried out with the analysis of their surface properties and weight swelling ratios. In order to analyze the surface properties of the crosslinked SBS gel and pH sensitive hydrogels, WCA and SEM were used. The dynamic swelling behavior of hydrogels demonstrated that, incorporation of SBS into the P(MAA-g-EG) hydrogels has a slight effect on swelling ratios when UV light is used. On the other hand, swelling ratios decreased significantly when visible light is used for polymerization of the hybrid hydrogels. Also, pH sensitive hydrogels that photopolymerized with visible light, maintained their integrity even at maximum swelling. In addition, release of PGB from pure pH responsive P(MAA-g-EG) hydrogel and its two types of composite forms were able to respond to environmental pH changes. Furthermore, both *in vitro* fibroblast viability

assay and *in vivo* implantation tests showed that pH sensitive hybrid hydrogels are not toxic and biocompatible. That's why, this developed approach would be meaningful for the improvement of controlled release formulations of PGB, using visible light cured P(MAA-g-EG) hydrogels. This would also provide sequential drug delivery from hybrid hydrogel network, due to their different hydrophobicities.

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