NANOGEL-INCORPORATING PH RESPONSIVE COMPOSITE HYDROGELS FOR CONTROLLED DRUG DELIVERY

GÜNCE EZGİ CİNAY

KOC UNIVERSITY
AUGUST 2015

Nanogel-incorporating pH Responsive Composite Hydrogels for Controlled Drug Delivery

by

Günce Ezgi Cinay

A Thesis Submitted to the
Graduate School of Sicences and Engineering
in Partial Fulfillment of the Requirements for
the Degree of

Master of Science
in
Chemical and Biological Engineering

Koc University
August 2015
Graduate School of Sciences and Engineering

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

Günce Ezgi Cinay

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Seda Kızılel, Ph. D. (Advisor)

Mehmet Sayar, Ph. D.

Nihan Nugay, Ph. D.

Date: 08/14/2015

ABSTRACT

In this study, both ultraviolet (UV) and visible-light have been used for the synthesis of novel hybrid pH responsive systems composed of poly(methacrylic acid-grafted-ethylene glycol), P(MAA-g-EG), and acryloyl group modified-cholesterol-bearing pullulan (CHPOA) nanogels. The system developed here has been utilized for controlled delivery of an anticonvulsant model drug pregabalin (PGB). The hydrophilic P(MAA-g-EG) network allows for pH dependent release of a loaded therapeutic agent, where CHPOA nanogel allows for the loading of a protein or hydrophobic drug into its structure. The hybrid hydrogels were synthesized under both UV and visible light, where bulk and surface initiated photopolymerization approaches were used. Bulk polymerized hydrogels were obtained through suspension of photoinitiator within the prepolymer solution, where uniform permeability properties were achieved for the synthesized pH responsive gel network. Surface initiated photo-polymerization was based on adsorption of photoinitiator onto an underlying substrate, where pH responsive hybrid gels with crosslink density gradients were obtained. Covalently attached nanogel within P(MAA-g-EG) hydrogel was characterized by Field Emission- Scanning Electron Microscope (FE-SEM). Swelling and release studies were carried out at pH 2.2 and 7.4 to mimic the physiological conditions in stomach and small intestine. Dynamic and reversible swelling experiments demonstrated higher swelling ratio profiles for visible light cured composite hydrogels. In addition, hybrid hydrogels incorporating with 5% nanogel, or synthesized with bulk photopolymerization demonstrated higher swelling. PGB release profiles at neutral pH demonstrated similarities for altered nanogel concentrations within the composite network. Biocompatibility of the hybrid network was also characterized using HeLa cell line *in vitro*. This approach for multifunctional membranes could be utilized for incorporation of specific molecules such as drug or protein with specific functionalities, so that sequential molecule delivery in response to specific stimuli could be achieved.

ÖZET

Bu çalışmada, poli(metakrilik asit-etilen glikol), P(MAA-g-EG) ve akriloil grubu ile modifiye edilmiş-kolesterol taşıyan pullulan (CHPOA) nanojel içeren özgün, pH'a duyarlı hibrit hirdojel sistemi, UV ve görünür bölge ışığı kullanılarak sentezlenmiştir. Geliştirilen bu sistem, antikonvülsan model ilaç pregabalinin (PGB) kontrollü taşınımı için kullanılmıştır. Jel sistemindeki hidrofilik P(MAA-g-EG) ağı yüklenen ilacın pH'a bağlı kontrollü salımını sağlarken, CHPOA nanojelleri kendi yapıları içerisine protein veya hidrofobik ilaçların yüklenmesine olanak sağlar. UV ve görünür bölge ışığı altında sentezlenmelerinin yanında hibrit hidrojeller, yığınsal veya yüzeyden başlayan polimerleştirme yöntemleri ile de sentezlenmişlerdir. Yığınsal polimerleştirme yönteminde fotobaşlatıcı, pre-polimer çözeltisinin içinde yer almaktadır ve sentezlenen pH'a duyarlı jel sisteminde tek tip geçirgenlik özelliği gözlenir. Yüzeyden başlayan foto-polimerizayon yöntemi ise, fotobaşlatıcının jelin sentezlendiği substratın yüzeyine adsorpsiyonuna dayanır ve pH'a duyarlı jel sistemi içinde çaprak bağ derişim farkı yaratılır. P(MAA-g-EG) içindeki kovalent bağlı nanojellerin karakterizasyonu, alan emisyonlu taramalı elektron mikroskobu (FE-SEM) ile yapıldı. Şişme ve ilaç salımı çalışmaları, fizyolojik mide ve ince bağırsak pH'ı göz önünde bulundurularak pH 2.2 ve 7.4'te yapıldı. Dinamik ve geri dönüşümlü şişme deneyleri sonucunda, görünür bölge ışığı ile sentezlenen hibrit jellerde daha yüksek şişme oranları gözlemlendi. Bunun yanında, %5 nanojel içeren hibrit hidrojeller ve yığınsal fotopolimerleşme yöntemi ile sentezlenen jellerde de yüksek şişme oranları gözlemlendi. Nötral pH'taki PGB salımı profilleri, jel ağı içindeki farklı nanojel konsantrasyonları için benzerlik gösterdi. Ayrıca, hibrit jellerin biyouyumluluğu, *in vitro* HeLa hücre hattı kullanılarak karakterize edildi. Çok fonksiyonlu membranlar için olan bu yaklaşım, farklı fonksiyonları olan, ilaç ve proteinler gibi belirli moleküllerin katılımı için kullanılabilir, ve böylece belirli uyarana karşı, birbirini izleyen sıralı molekül taşınımı gerçekleştirilebilir.

ACKNOWLEDGEMENTS

Apart from my efforts, the success of this study depended mainly on the encouragement and guidelines of many others. First, I would like to express my appreciation to my thesis advisor Assoc. Prof. Dr. Seda Kızılel for her continuous support, encouragement and superior guidance throughout this study. This dissertation would not have been possible without her valuable advices and help. Her continuous enthusiasm for science and constructive positive critisim throughout these two year was a priceless motivation for me. I sincerely appreciate for accepting me in her group, and introducing me with true scientific environment where I get invaluable insights about my career.

Next, I would like to thank my committee members, Prof. Dr. Nihan Nugay and Assist. Prof. Dr. Mehmet Sayar for participating in my thesis committee. I deeply appreciate their useful comments and contributions to my thesis.

I would like to thank Scientific and Technological Research Council of Turkey (TUBITAK) for M.Sc. scholarship. I am also thankful to our collaborator Assoc. Prof. Dr. Yoshihiro Sasaki from Kyoto University for providing the nanogels that I used in my research. I am grateful to Natali Çizmeciyan since she provided the assistance with UV light applications. I also acknowledge Barış Yağcı and Cansu Yıldırım, who are the staff of the KUYTAM (Koc University Surface Science and Technology Center), for their help to perform FE-SEM and UV-Vis spectrophotometer analysis. I thank to Abdi İbrahim Pharmaceutical Company for providing Pregabalin.

In addition, it was a great pleasure to be one of the members of our laboratory group. I wholeheartedly thank to my research group friends, Tuğba Bal for introducing me to cell culture and for her warm welcome to all my questions and requests with her scientific mind and knowledge during my master, Pelin Erkoç for her motivating smiling face, continuous support, and for the unforgettable memories we shared in Italy, Derya Aydın for her friendship and delighting conversations, Aysu Okur for her motivating and stimulating behavior, Sadi Gürses for the indelible imprint he left in my life with his great personality, and Mohammad Alipour for his consistent positive attitude and energy. I also send my sincere

regards to my graduated lab friends Caner Nazlı, Özlem Çevik, and Mohammad Rezamohammadi for their friendship, support, advices and encouragement, in addition to our enjoyable lab hours. I wish all the best positions in their lives. I would like to thank especially Özlem Çevik, for the extensive lab training she gave before she graduated and her continuous support in all my experiments.

I also would like to thank my beloved friends Bahar Temizer and Nisa Nildan Dilaver who usually know me better than I do, share all our childhood and youth, and stay with me anytime I need since the first year of primary school. I also thank Sadi Gürses, Pelin Erkoç, Göktuğ Gönel, and Sinan Yücetürk for all times that I feel like I am seizing the moment, living life to the fullest, and adding value to my life with their greater friendship. I also would like to thank my flat mates Irina Crivet and Özlem Ünal for their strong and heartfelt support, positive personality, and for the memorable moments we shared in our home.

Finally, I would like to thank to my family for their everlasting love, solid support in all of my efforts, understanding, patience, motivation and encouragement. They are the ones who made me the person I am today. I am grateful to them for ensuring me that we are a family and they will be there in any condition.

Without them, I could not be here.

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NOMENCLATURE

Ct	Concentration of PGB in the release solution at time *t*
q	Mass swelling ratio
Ws	Weight at swollen (wet) state
Wd	Weight at dry state
pKa	Acid dissociation constant
M_t	Mass released at time *t*
M_∞	Total weight of PGB released during the release experiment
V	Total volume of release solution
Vs	Sample volume that is taken in release experiment

Chapter 1

INTRODUCTION

The main goal of many of the pharmacological treatments is to administer the pharmaceutically active agent in a desirable amount to produce a therapeutic effect with tolerable side effect and toxicity. Pharmaceutical agent should remain in the therapeutic window, between the sub-therapeutic level and the toxic level of the drug, for a desired period of time to produce the curing effect. For efficient and targeted delivery of drugs, novel drug delivery system (DDS) design is needed [1]. Progress in material science, (polymer) chemistry, biotechnology, and nanoengineering fields significantly contributed to drug delivery field over the past few years. From polymers and liposomes, to micelles and antibodies, various DDSs have already investigated and several of them have been used clinically, today [2].Development of functional and sensitive polymeric network structures as controlled drug delivery vehicles demonstrates significant potential for sequential release of biomolecules, such as protein and peptide therapeutics [3], and other drugs in case of specific stimuli [4]. Thus, hydrogels have been extensively used in biomedical and pharmaceutical applications including drug delivery systems [4]. Hydrogels are water swollen networks that have created great interest due to their several characteristics, such as their biocompatibility, high water content, and their chemical and mechanical similarity to the natural environment of cells. They have been considered for the delivery of bioactive molecules, cells, and as

dental materials and biosensors [5-7]. Especially, stimuli responsive hydrogels have capacity to deliver therapeutics to the target site with a stimuli responsive manner, and hence these network systems are promising for different types of biomedical applications [8, 9]. Also, depending on their unswollen and hydrated states, stimuli sensitive hydrogels can exhibit favorable physicochemical features [10, 11]. These properties significantly change with response to environmental alterations, such as temperatures, pH, electric field, magnetic field, ultrasound, wavelength of light, and ionic strength [9, 12, 13].

PH-responsive hydrogels are a subset of stimuli-responsive systems that respond to perturbations in the environmental pH changes, due to the presence of pendant ionizable groups in the polymer backbone [7]. These systems attract significant interest for their use in different locations in the body exhibiting substantial pH changes, during either normal function, as it is seen the gastrointestinal tract [14], and vagina [15], or as part of a disease state, as it occurs in the extracellular tumor environment [16].

Methacrylic acid grafted with poly(ethylene glycol) P(MAA-g-EG) hydrogels have been frequently used anionic polymeric networks which respond to pH changes in the environment. Carboxyl groups within the polymer structure of anionic pH responsive hydrogels, as in the case of the weak polyelectrolyte poly(acrylic acid) (PAA) [17], ionize in alkaline buffers. With the formation of electrostatic repulsive forces, the water can be absorbed by the hydrogel network, and swelling is observed [7, 18]. Previously, UV light exposure was used for the synthesis of pH sensitive hydrogels via free radical photopolymerization [17]. Even though the synthesis of pH responsive hydrogels with UV exposure has significance in their use in biomedical applications, biocompatible and responsive hydrogels have also been synthesized through visible light exposure. Recently, visible-light-mediated polymerization

has been used for the fast synthesis of pH responsive hydrogels with lower amounts of photoinitiator in our group [19]. The study involved the synthesis of pH sensitive P(MAA-g-EG), and SBS incorporating P(MAA-g-EG) hybrid hydrogels via visible light photopolymerization [20], where the release of a model drug from this hybrid hydrogel system was examined [21, 22].

Over a few decades, nanogels have attracted increasing attention for the delivery of biomacromolecules, imaging agents, and different therapeutics [23]. Sekine et al. reported the synthesis of physically cross-linked cholesterol-bearing pullulan (CHP) nanogels, where cholesteryl and pullulan groups constitutes the hydrophobic and hydrophilic components, respectively [24]. CHP nanogels have various advantageous properties, such as their ability to carry bioactive molecules including DNA, RNA, and proteins in their 30 nm diameter space, and their artificial molecular chaperone function [25]. CHP nanogels have been further engineered to develop functional hierarchical nanogels by the same research group, where bottom-up assembly of acryloyl group bearing CHP nanogels and acryloyl group modified-cholesterol-bearing pullulan (CHPOA) nanogels were synthesized [26, 27].

Controlled delivery of anticonvulsant drugs, specifically PGB, is important for the treatment of different chronic conditions, such as epilepsy [28, 29], general anxiety disorder (GAD) [30], and neuropathic pain [31]. PGB is a unique Food and Drug Administration (FDA) approved neuropathic pain medication, specifically for the treatment of diabetic peripheral neuropathy [32]. Besides its clinical use, extended release of PGB from a drug delivery vehicle has not been studied until the study of our group, where pH responsive release of PGB was obtained from bare and SBS hybridized P(MAA-g-EG) hydrogels [33, 34]. In this study, acryloyl group modified-cholesterol-bearing pullulan (CHPOA) nanogel

incorporating pH responsive hybrid hydrogel systems were developed for controlled release of an anticonvulsant model drug, PGB. P(MAA-g-EG) was chosen as hydrophilic and pH sensitive component [20], where acryloyl beaing CHPOA nanogels were incorporated into this network to allow the loading of a high molecular weight and hydrophobic protein into the hybrid hydrogel network [25]. Nanogel incorporating stimuli sensitive hydrogels were synthesized through both UV light and visible light induced photopolymerization. In addition, the synthesis of these gels were also performed through different polymerization approaches; as *(i)* bulk polymerization in which the photoinitiatorwas suspended within the prepolymer solution, and *(ii)* surface initiated polymerization in which the photoinitiator was physically adsorbed onto a substrate surface where the polymerization reaction initiated from the surface. Furthermore, the effect of altered nanogel concentration on swelling and PGB release profiles of hybrid hydrogels were investigated.

In this thesis, chapter 2 presents an overview on recent investigations in drug delivery systems and stimuli responsive hydrogels. Information about pH sensitive P(MAA-g-EG) hydrogels and CHPOA nanogels have been provided, and synthesis of nanogel incorporating 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 presents materials and methods as an experimental part of this thesis. In this chapter, synthesis of P(MAA-g-EG) hydrogel, and differently concentrated CHPOA nanogel incorporating hybrid P(MAA-g-EG) hydrogels was accomplished through UV and visible light photopolymerization. The synthesis for different sets of hydrogels were also performed with both bulk and surface initiated photo-polymerization approaches. Surface

characterization of alteredhydrogels was investigated through FE-SEM images. Swelling experiments and PGB release experiments were carried out under low and high pH conditions for all types of P(MAA-g-EG) hybrid hydrogels. Also, *in vitro* HeLa cell survival assay, important for biocompatibility of the hybrid hydrogels, was presented in the same 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 discusses the potential of this system for future studies.

Chapter 2

LITERATURE REVIEW

2.1 DRUG DELIVERY SYSTEMS

The main requirement for pharmacological treatments is to administer the active agent in a desirable amount to produce a therapeutic effect with minimum side effects and toxicity. The pharmaceutical agent is needed in sufficient amount when it reaches the target site and it should also reside in target area for sufficient amount of time to achieve the desired therapeutic effect. Several issues are needed to maximize the therapeutic effect and minimize side effects. Traditionally, independent from the route of delivery, the active agent reaches the blood stream, not directly to the target site, and it should be protected from enzymes and various other barriers to reach the target site. Many of the treatment strategies do not target the disease site, and hence higher doses of drug have been administered, where only some amount of the drug is expected to reach the target tissues or cells. In addition, active substances such as genes, enzymes and peptides are not able to withstand the blood circulation because of their lower stability and physicochemical properties. Therefore, efficient design of delivery vehicles is desirable for successful delivery of active molecules towards target site. With the new designs of drug delivery systems (DDSs), it is possible to achieve sustained release of drugs at a targeted site [35].

In the past few decades, with the recent progress made in polymer and material science, nanoengineering and biotechnology, drug delivery field has grown. Various DDSs are already available in forms of polymers, liposomes, micelles, antibodies or proteins and more diverse options have been investigated, such as viruses and nanobodies, or iron oxide, and silica

nanoparticles. Today, clinically well established formulations have been routinely used after their years long evaluation and optimization in animal models and in patients [36].

2.1.1 Drug Delivery Systems According to the Route of Administration

The administered drug can directly enter into the body, or the entry can be achieved by overcoming the skin or the mucosal membranes [37].

2.1.1.1 Drug administration through injection (Parenteral drug delivery)

Aqueous solutions or the dispersed phased (having particles smaller than 100-150 nm) dosage forms are usually used for intravenous delivery. In addition, the dermal dosage forms are also called as topical delivery systems because their affect does not give systemic activity but it has a local affect. The drug administration through direct injection or infusion is termed as parenteral drug delivery and it is classified into several subgroups depending on the site of administration into the body [37].

Subcutaneous injection

The needle is inserted into the fatty tissue just under the skin and at most 2.5 mL of the formulation is applied to the injection site. Insulin and morphine are commonly administered via this route.

Intramuscular injection

Up to 5 mL dosage forms are administered into the muscles in through this route.

Intravenous injection

The precise volume of the dose is directly inserted into a vein by a needle. Single dose injection for small volumes and infusion for larger volumes can be preferred.

Intradermal injection

Injection is given directly into the skin as it is used in allergy skin testing.

Intraperitoneal injection

Injection is done through the peritoneum which is the thin, transparent membrane that lines the walls of the abdomen.

2.1.1.2 Drug administration through the skin

While parenteral delivery routes use aqueous or oily dosage forms, for the drugs administered through the skin, generally semisolid dosage forms are preferred such as gels, creams and pastes. In transdermal controlled drug delivery systems, the main aim of the formulations is to allow skin to absorb the dosage, not to prevent particles from entering, as this is the main function of the skin. Hence, penetration enhancers are added and the dosage form controls the uptake into the skin [38].

2.1.1.3 Drug administration through mucosal membranes

Drug administration through mucosal membranes is the most important drug delivery route because of the superior permeability of the mucosal membranes compared to skin, such as the small intestine that is specialized for absorption. The most commonly used and most convenient drug delivery route is the oral route which includes entry through the mucosal membranes of the gastrointestinal tract [37].

Easy administration of drugs, patient compliance, and cost effective manufacturing are some of the advantages of oral drug delivery [39]. In addition to gastrointestinal tract, the buccal, sublingual, rectal, vaginal mucosa and the lung and nasal mucosal membranes act as absorption sites. Although oral drug delivery is the most convenient route for drug delivery, several issues should be considered, such as difference of gastric residence time from patient to patient, and of the clearance time of the liquid or solid dosage forms from the stomach. Additionally, the difference in pH from stomach (1.5-2) to small intestine (4 to 7) may affect the stability of the ionization degree of the ionizable drugs and their absorption from the small intestine. Also, after absorption drugs are transported to the liver and the gut to be metabolized before going into general circulation, and if the drug is susceptible to degradation it may lose its activity, which is called as heparic first-pass effect. On the other hand, for the delivery of certain drugs, delivery through other mucosal membranes such as vaginal, buccal and nasal delivery, may be preferred as in this case the drug is distributed in the body before reaching the liver [39].

2.1.2 Drug Delivery Systems According to Their Mechanism of Drug Release

Another way of differentiating drug delivery systems is the way the drug is released. By dosage forms, the release rate and the location is controlled. According to this systematic defined by FDA, they are classified into two, as immediate-release and modified-release dosage forms. Modifications in drug release profiles are beneficial for the optimizations in terms of the stability, safety, efficacy and therapeutic profile of a drug. Modified-release systems are also desirable to increase the patient compliance and convenience of administration [37].

2.1.2.1 Immediate release

It allows the immediate release of drug after its administration and dissolution in gastrointestinal tract, without any delaying or prolonging. The onset of action is fast and so, it is useful for various therapeutic reasons, such as pain relief. In the cases of intravenous injections and infusion, the drug is already in solution and no time is needed due to permeation through the skin or mucosal membranes, so the pharmacological effect may be seen in seconds. The duration of the onset of action increase from orally taken drugs in solution to drugs in forms of powders, granules, tablets, and capsules [37]. For immediate release dosage form, the drug is release at once and follows a first order kinetics profile.

2.1.2.2 Modified release

The release of the drug starts sometime after its administration and it may occur for a prolonged period of time. This release system is further divided into delayed-, extended- and targeted-release systems [37].

2.1.2.2.1Delayed release

The release of the drugs does occur at a time but not immediately after their ingestion. These systems may be used to protect drug from low pH environment of stomach or to protect

stomach from irritation, and allow the drug to be release in small intestine. For this aim, generally polymer coatings have been used [37].

2.1.2.2.2 Extended release

The formulation of extended-release products is optimized in order for them to be available in the body for a prolonged period of time after their oral administration. These systems help lower dosing frequency compared to conventional dosage forms. Prolonged release profiles and reduced frequency of dosing are achieved by the use of extended-release. This is especially helpful to chronic diseases requiring lifelong drug usage. Extended release is possible using sustained- or controlled-release dosage forms. In these systems, the rate of drug release is maintained over a sustained period [37].

2.1.2.3 Sustained release

Sustained-release dosage forms are only applied to the oral drug delivery. Sustained release can be achieved through the use of polymers in two ways [7];.

2.1.2.3.1 Reservoir delivery systems

The granules or tablets are coated by hydrophobic and non-degradable polymers in this area. These systems show linear release as a function of time (zero-order kinetics) [37]. The reservoir structure has some advantages, such as constant biomolecule release rates and long circulation life due to the protective effect of the reservoir [40].

2.1.2.3.2 Matrix delivery systems

In matrix systems, the polymer is used to form a matrix which contains the drug in dispersed or dissolved form. Different from reservoir systems, matrix systems follow a linear release as a function of the square root of time. Matrix delivery systems are more proper for the delivery of therapeutic agents, large molecules, or proteins, because of the limitations of reservoir systems [37]. These systems are commonly used because of their better performance, cost-effectiveness, and ease in development [7].

2.1.2.4 Controlled release

In addition to the sustained release profiles, these systems allow to constant predictable plasma concentration independent from the application site [40]. Controlled-release dosage forms can be used in various routes from transdermal to oral and vaginal administration. The release kinetics is generally zero-order. Ideally, the release rate from controlled release systems should be equal to the absorption rate of the drug [37]. Reduced dosing frequency, decrease in dosage, consistent level of drug concentration in circulation, advanced patient compliance and attenuated toxicity are among the benefits of controlled drug delivery systems over other conventional methods [41].

2.1.2.5 Targeted release

Targeted delivery systems are needed to control the biodistribution of the drug by exploiting the characteristics of the drug target and the drug carriers. This system is necessary over the controlled release dosage form because once the drug released there is very little control over the drug distribution, and due to non-specific targeting reduced therapeutic efficacy and increased toxicity/side effects are observed. Through specific targeting, the distribution of the drug in the body is controlled in tissue, cellular or subcellular levels [37].

In drug delivery field, hydrogels have gained a considerably extensive interest due to their unique properties. For both pharmaceutical and biomedical applications, the use of hydrogels as drug delivery vehicles is promising [7].

2.2 HYDROGELS

For the synthesis of idealized drug delivery systems, wherein the desired amount of pharmaceutical active agent is available in the site of action when required, various natural and synthetic biomaterials have been proposed for years [7].

Since 1960, when hydrogels are proposed to be used in contact lenses [42], their use in biomedical[6] and pharmaceutical [8] applications has extended exponentially.

Hydrogels are a cross-linked network of hydrophilic polymers and firstly, PJ Flory (1944–1952; Nobel prize 1974) set the basic theories for the analysis of hydrogels regarding their thermodynamics, critical miscibility characteristics, and the statistical mechanical analysis [43].

2.2.1 Physical Structure

As the name suggests hydrogels have high water content and a soft structure. Their hydrophilic nature encourages the uptake of water and allows the solid yet hydrated structures, much like cells. Due to these physcial properties, compared to other synthetic biomaterials hydrogels resemble living tissues closely and they are ideal candidates for their use in tissue engineering, implants, drug delivery, and more [7].

Hydrogels are cross-linked insoluble networks. The cross-links may be in differential forms, such as noncovalent bonds, physical entanglements, dipolar interactions, ionic bonds or as covalent chemical links [44].

Hydrogels demonstrate low interfacial tension; therefore their tendency to absorb proteins from body fluids is minimal. They have the ability to absorb large amounts of water (>20% of its weight of water) and swell without losing their 3D structure. They are stable water insoluble networks due to the presence of chemical or physical cross-links [45]. With the use of various monomers, hydrogels posses differential sized pores, allowing different sized biomolecules to be loaded and released [7].

2.2.2 Hydrogel Synthesis

Either by simultaneous polymerization of monofunctional monomers and cross-linking with polyfunctional monomers [46] or by cross-linking of linear polymers [47], hydrogel based drug delivery systems can be prepared. The adjustment of the mechanical strength of the hydrogels can be achieved by adjusting the cross-linking density [48]. Among the most commonly used hydrogels, poly(ethylene glycol) (PEG) and poly(vinyl alcohol) (PVA) can

be given as the examples of synthetic hydrogels and collagen, hyaluronan, agarose, chitosan and fibrin as the naturally occurring ones. The use of different monomers in hydrogel synthesis and their spatial order designate the final characteristics of hydrogels, such as their morphology. They can be found in amorphous, semicrystalline, or colloidally aggregated forms [44].

Chemical cross-linking of the hydrogels can be achieved through many methods. For instance, use of dimethacrylates as monomers in chain polymerization leads to the incorporation of the polymer backbone at different cross-linking points to the two methacrylate functionalities [43].

In addition to different cross-linking methods used, the hydrogel structure significantly changes by the other synthesis parameters, such as the used monomers and their percentages, solvent selection, or the previous mechanical and degradative happenings to the polymer [49]. Depending on the route of administration, the hydrogel-based dosage forms are designed by cross-linking the polymers in a desired shaped mould [7].

Hydrogel formulations which can perceive and transmit stimuli and respond by exhibiting pyhsical or chemical changes, are named as smart hydrogels [50]. These stimuli sensitive gels change in their phases, surface energies, shapes, permeation rates and so on, resulting in the controlled release of imprisoned drug [13].

Depending on the monomers incorporating in the hydrogel network and the conditions of the medium, such as pH differences, hydrogels carry different charges of a full spectrum from cationic to neutral and ampholytic. These differential charges on the hydrogel structure depend on the protonation status of incorporating pendant groups. Generally, the cationic properties of hydrogels imparted from the amine pendant groups and the anionic properties from the carboxylic acid pendant groups [43].

Hydrogels are generally glassy before they get hydrated, and following the water absorption, desorption of the drug occur [51]. The drug delivery rate is mediated by the resistance of the polymer against the volume increase or the change in the shape [47]. In water, the dehydrated hydrogel allows the water molecules penetrate in the polymer network. With the entry of enough water, the radius of gyration and the stress on the polymer chain elevated and a macroscopic swelling is observed [7].

2.2.3 Commercially Available Hydrogels

The use of hydrogels is not restricted to drug delivery only. Hydrogels have been used in other biomedical applications, such as their use in surgical and breast implants, contact lenses, wound dressings, biosensors, surgical catheters and so on [6, 18, 52]. Several hydrogel-based drug delivery systems are commercially available, such as surgical aids and over-the-counter dressings, and most of them are patent protected or have trademarks, such as Hypan® used in contact lenses, Aquamere™ used in oral drug delivery or Cervidil® vaginal insert used for the initiation and/or continuation of cervical ripening [7].

2.2.4 Stimuli-responsive Hydrogel Systems

Ideally, drug release profiles from a drug delivery system should be synchronized with deviations in the physiological conditions. The design should sense the changes in the body and alter the drug desorption accordingly [7].

In response to both internal and external stimuli, hydrogels show significant changes in their network structure, swelling behavior, mechanical strength and permeability [18]. To modulate drug release from hydrogels, temperature, pH, chemicals, shear stress, electric fields, and light can be used (Figure 2.1) as it is documented in the literature [7, 53]. Several stimuli responsive hydrogels are known to a shape memory and they can return back to their original shape after the stimuli has stopped, due their elastic deformability characteristics [54].

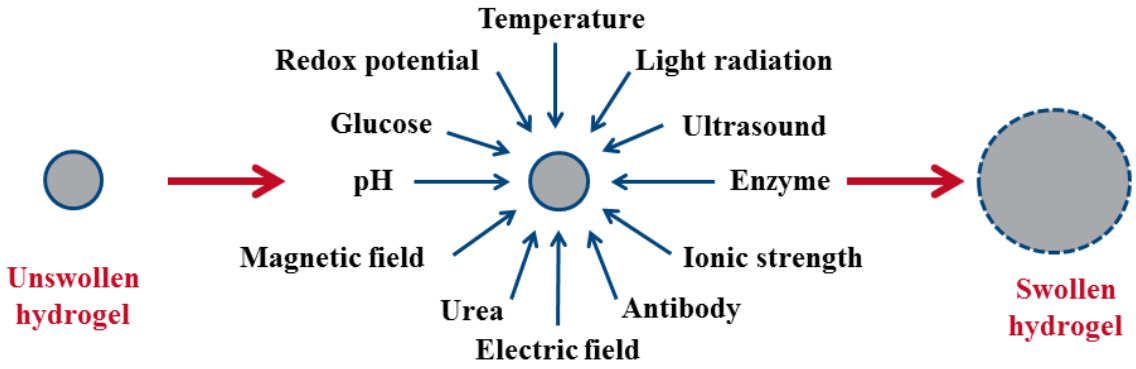

Figure 2.1 Schematic representation of types of stimuli which cause alterations in hydrogel structure.

Moreover, in pathological conditions where more than one stimulus is present, there is a need to design new release systems that are able to release the drug in the presence of both stimuli rather than a single one. For instance, poly (***N***-isopropylacrylamide) PNIPAAm (temperature-sensitive) and PAA (pH-sensitive) composite hydrogels show both pH- and temperature-sensitive swelling behavior [55]. This dual sensitive system has already been proposed their use for the delivery of streptokinase and heparin, calcitonin, and insulin [7].

2.2.4.1 pH responsive hydrogels

Internal stimuli-responding systems have been studied more because of their feasibility in therapeutic applications, cost and product scale-up considerations. Among them, pH-responsive systems has been extensively studied for their use in several body parts exhibiting substantial pH variations, during either normal function such as gastrointestinal tract [56] and vagina [15], or as part of a disease state, such as blood vessels (in certain cardiovascular defects) [57], inflamed tissue/wounds [58], and the extracellular tumor environment [16].

2.2.4.1.1 Structural framework

pH-responsive hydrogels are a subset of stimuli-responsive systems that respond to perturbations in the environmental pH changes. The pH responsive behavior of the hydrogel

network is achieved by the presence of ionizable pendant groups in the polymer backbone [7]. In Figure 2., the structures and ionized forms of anionic and cationic polyelectrolite examples are shown [59].

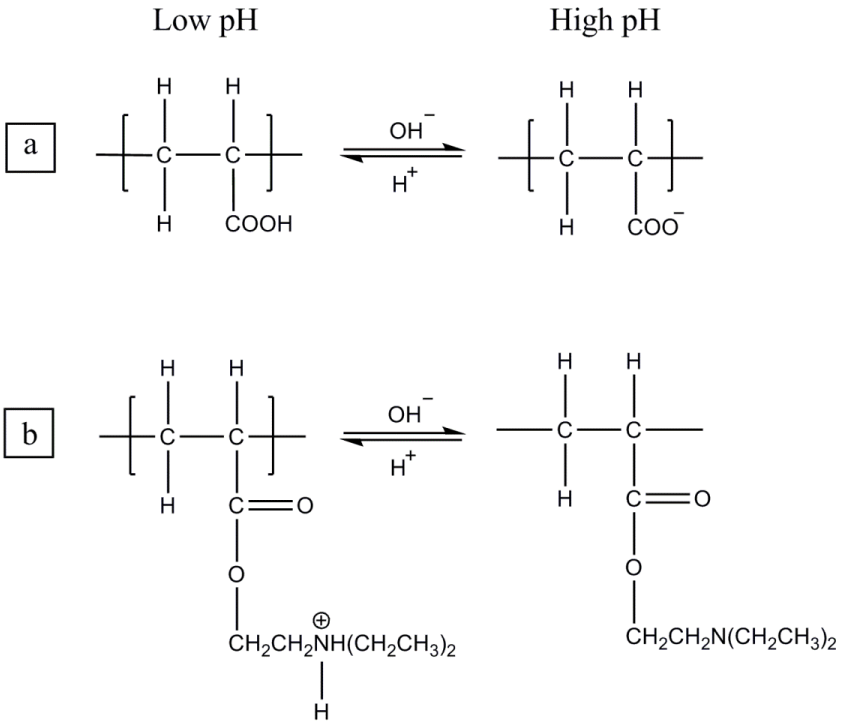

Figure 2.2 The structure and the ionization of (a) PAA as an anionic polyelectrolyte, and (b) PDEAEM as a cationic polyelectrolyte.

The pendant groups ionized in mediums of appropriate pH and ionic strength, and swelling or deswelling occurs according to either the repulsive or the attractive forces generated [13]. With the change in the mesh size of pH-responsive network, the drug release is controlled. Poly(methacrylic acid) (PMAA), poly(acrylamide) (PAAm), poly(dimethylaminoethyl methacrylate) (PDMAEMA), and poly(acrylic acid) (PAA) are among the most commonly studied ionic polymers for pH-responsive hydrogels [18]. The pH-responsive hydrogels are categorized into two, as anionic and cationic, depending on the ionizable pendant moieties on their backbone structure [60].

2.2.4.1.1.1 Anionic hydrogels

In the case of anionic hydrogels, the pendant functional groups are only ionized at a pH above the acid dissociation constant, p$\boldsymbol{K}_a$ of the polymeric network, and because of the presence of ions and generation of the osmotic swelling force hydrogel swells [7]. The anionic hydrogel remains collapsed at low pH due to the tight physical interactions within the network. With the increase in pH greater than the p$\boldsymbol{K}_a$, the network dissociates and the polymer swells [5].

2.2.4.1.1.2 Cationic hydrogels

These hydrogels were comprehensively studied by Siegel et al. [61], where copolymers of cationic monomers, such as methyl methacrylate (MMA) or dimethylaminoethyl methacrylate (DMAEM) were used. Cationic pendant groups are ionized at a pH below their pK_a and the network swells at pH < p$\boldsymbol{K}_a$ [43]. The swelling behavior of pH responsive anionic or cationic hydrogels is different in acidic and alkaline buffer solutions [7], as shown in Figure 2.3.

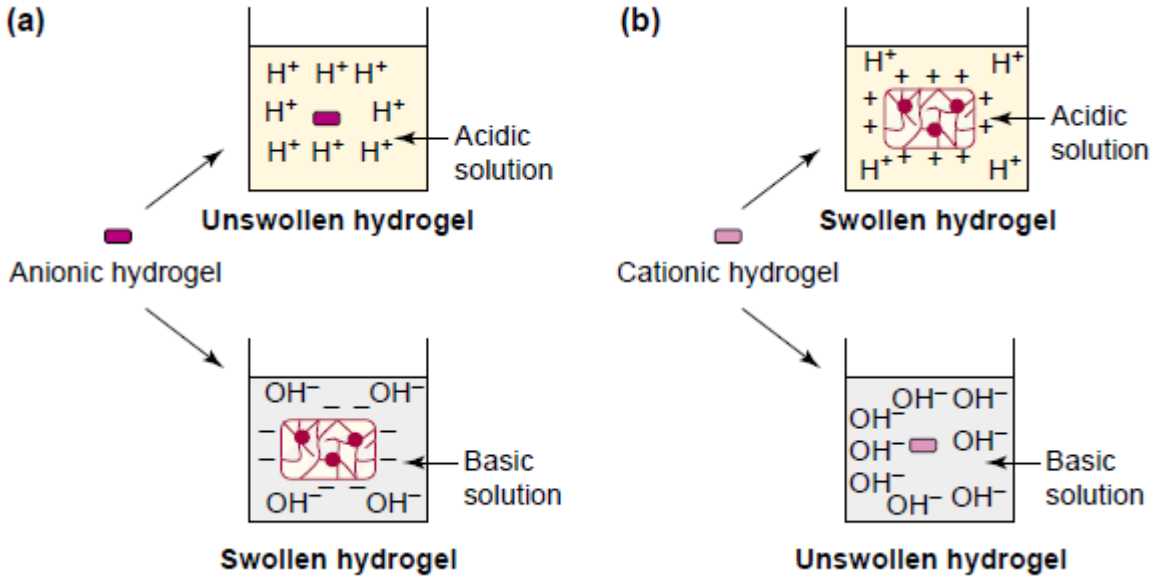

Figure 2.3 pH swelling behavior of (a) anionic, and (b) cationic hydrogels.

2.2.4.1.2 Factors influencing pH-responsive swelling and drug release behavior

The swelling degree of ionic polymers is influenced by the factors related to the properties of the polymers or the swelling medium. The factors related to the polymer properties can be listed as the cross-linking density, hydrophobicity/hydrophilicity, degree of polymerization and the charge, p$\boldsymbol{K}_a$, and concentration of the ionizable group. In addition, ionic strength, pH of and the counter ion in the swelling medium influence the degree of swelling of ionic polymers [62].

2.2.4.1.3 Drug release profile

Design of the pH-responsive drug release systems can result in a monophasic or pulsatile profile. Uniform drug release correlated with the pH gradient through the gastrointestinal lumen is needed in peroral controlled delivery systems. Albumin cross-linked 1-vinyl-2-pyrrolidinone hydrogels exhibited increased swelling at intestinal pH, allowing maximal transmission of the drug [63].

For the cases of diseases showing rhythmic patterns, pulsatile pattern of drug release is required. A poly(***N***-isopropyl acrylamide-co-methacrylic acid) hydrogel was studied for the localized delivery of heparin and streptokinase in response to temperature and pH pulses and a pulsatile swelling behavior is observed [57].

2.2.4.1.4 pH responsive hydrogels for controlled drug delivery

pH-responsive hydrogel systems have been used for the controlled delivery of various therapeutic drugs, such as chemotherapeutics [64], genetic material like RNA and DNA (electrostatic binding to the charged hydrogel network) [65], and proteins [66]. Loading of these bioactive molecules is achieved by the encapsulation or dissolvation of the therapeutics within the hydrogel [67]. The controlled release of the drug from the hydrogel network is triggered by the variations in the environmental pH, such as the pH change through the digestive system or intracellular trafficking pathways [68].

Among the various applications of pH-sensitive hydrogels, a saturated insulin solution immobilized hydroxyethyl methacrylate-based copolymer has been used for insulin delivery. The system is triggered by the diffusion of glucose into the hydrogel wherein glucose oxidase and catalase were localized, and the glucose entry results in the enzyme-catalyzed conversion of glucose to gluconic acid, and the following pH decrease leading to swelling. The glucose concentration decreases due to the insulin release and the hydrogel returns back to its non-swollen phase, which leads to the self-regulation of the drug delivery device, the decreased rate of insulin delivery [69].

The pH dependent swelling of the anionic hydrogel is triggered by the substantial pH change in the gastrointestinal tract, ranging from pH 1.0 in the stomach to upwards of pH 8 in upper small intestine and colon [14, 70]. In acidic pH environment as low as pH 1.0, the anionic hydrogel remains in the collapsed configuration, and the drug is therefore protected from being denatured by the harsh stomach environment. Afterwards, in certain areas in small intestine and colon following the increase in the environmental pH, the hydrogel swelling is initiated [43].

2.2.4.1.5 P(MAA-g-EG) Hydrogels

Specifically, a copolymer consisting of methacrylic acid (MAA) polymer backbone and grafted poly(ethylene glycol) (PEG) chains, P(MAA-g-EG), has studied for the oral delivery of biomolecules to the upper small intestine by Peppas and associates. MAA is the pH-responsive part of this anionic hydrogel network due to its pendant carboxylic acid groups which are protonated below its pK_a of 4.8 and deprotonates at pH > p$\boldsymbol{K}_a$. With the protonation, MAA undergoes complexation through hydrogen bonding ith the etheric oxygen of the PEG, and thereby the hydrogel remains in its collapsed state. At higher pH values, negatively charged pendant carboxylic acid groups are deprotonated and the following electrostatic repulsion starts the swelling behavior. Herein, due to the hydrophilic nature of PEG, swelling rate for water increases [71]. Incorporation of high molecular weight PEG tethers into the P(MAA-g-EG) network has a greater importance due to the mucoadhesive behavior of PEG. The tethers of PEG interact with the mucus lining of the upper small intestine and penetrate into the polysaccharide matrix of the mucus, resulting in the engagement of the physical

entanglements and hydrogen bonding [72]. Due to this characteristics of the hydrogel, it anchors at the intestinal wall where it remain longer and can ensure complete diffusion of the therapeutic out of the matrix [73, 74].

The P(MAA-g-EG) network has been successfully used for the oral delivery of several protein therapeutics, such as insulin, interferon-b, and calcitonin [43]. The evaluation and optimization of P(MAA-g-EG) and other hydrogel networks for a specific therapeutic of interest is the upmost importance. The loading efficiency and release kinetics of the hydrogels are substantially affected by the small changes in the isoelectric point, hydrophobic/hydrophilic composition and size of the hydrogel [43].

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

Generally, the synthesis of pH sensitive P(MAA-g-EG) hydrogels have been achieved by free radical photopolymerization under UV light where Igracure has been used as a photoinitiator [17]. For instance, Schoeneret al. synthesized P(MAA-g-EG) hydrogels with poly(methyl methacrylate) (PMMA) nanoparticles [64]and amphiphilic interpenetrating networks (IPNs) of P(MAA-g-EG) [75] through UV light mediated polymerization.

Synthesis of P(MAA-g-EG) hydrogels using UV light has significant importance for the production of biocompatible and responsive hydrogels, however these biomedical products can also be obtained through visible light exposure. Moreover, visible-light-induced photopolymerization process allows for fast polymerization, with less amount of initiator, compared to the UV light-induced polymerization [19]. Kizilel et al. [76, 77] previously showed the visible-light-induced photopolymerization of PEG diacrylate hydrogels. In this study, P(MAA-g-EG) hydrogel synthesis was carried out by using the same photoinitiator, eosin Y, and the co-initiator, TEA, to follow the same photoinitiation mechanism. Eosin Y was preferred as the photoinitiator due to its convenience based on its spectral properties with an argon ion laser. The purpose of choosing TEA as a co-initiator, is its property to donate an electron and a proton donor, which result in the formation of alpha-amino radical. This primary radical initiates polymerization and further cross-linking reactions. The details of the visible light (514 nm wavelength) induced photoinitiation process can be followed in Figure 2.4 [76].

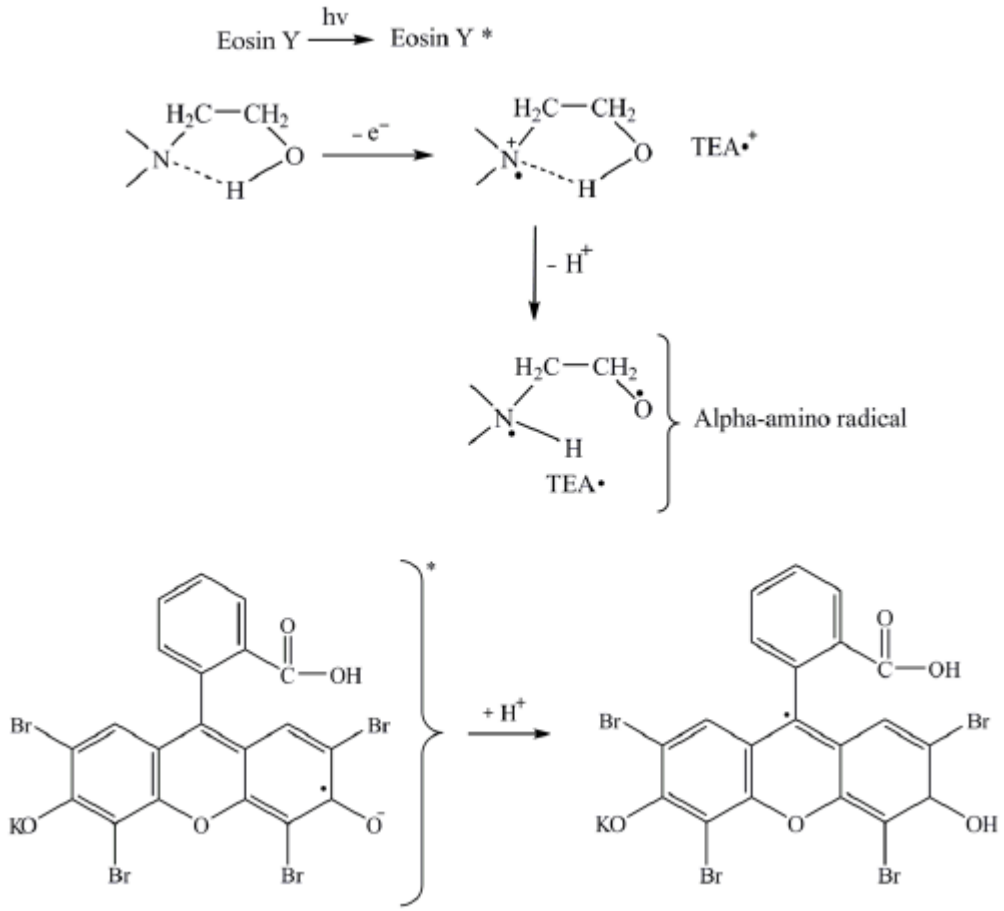

Figure 2.4 The initiation mechanism by eosin Y and TEA.

Thus, the main difference between the previous UV light and current visible light induced pH sensitive hydrogels synthesis is the addition of eosin Y and TEA instead of Irgacure.

2.2.5 Hybrid Hydrogels

The term composite or hybrid hydrogels refer to a hydrogel network which includes particulate systems [78], or the hydrogel systems in combination with polymers [79]. Hybrid hydrogel systems have been recently developed and have possible usage as advanced drug delivery system [80]. Hydrogels are commonly preferred for the delivery of hydrophilic drugs. According to the demands on hydrophobic drug delivery, such as the low drug loading efficiency and limited release of the hydrophobic drugs from the gel network, synthesis of

composite delivery systems retaining both hydrophilic and hydrophobic properties together, is a hot topic [81].

Hydrogels have been used for the delivery of hydrophobic drugs by using various strategies, such as the incorporation of hydrophobic domains into the polymeric system [82] or the combination of hydrophobic nanoparticles with hydrogel [17]. In a study of Men et al., thermo-sensitive Pluronic hydrogel was combined with the cationic nanoparticles and the delivery of hydrophobic anticancer drugs using this system was investigated [83]. In another study, Giray and associates utilized PEG hydrogel coated hydrophobic silica aerogels for the release of a model drug ketoprofen [84].

Different from the hydrogel-polymer systems or networks including particulate systems, synthesis of hydrophobic polymer chains grafted on hydrophilic polymers is another approach to create composite structures. These systems have superior properties, such as their excellent tissue compatibility, and their mimicry to human tissue. However, they are poor in mechanical means upon swelling [85], and Hsiue et al. built a hydroxyethyl methacrylate (HEMA) grafted SBS polymer system to address this problem, and concluded in better mechanical properties of the composite compared to poly(HEMA) [86].

Functionalization of hydrogels with various materials, such as polymeric microspheres [87], liposomes [88, 89], micelles [90], or nanogels [91, 92] have already been investigated for their use in tissue engineering scaffolds and implanted drug delivery systems. Thus, developments of composite systems have potential for a variety of biomedical applications. For their use in the delivery of therapeutics, by the alterations in the building blocks of the composites, the biomolecule release profiles can easily be modified.

2.2.5.1 Synthesis of nanogel incorporating hybrid hydrogels

From the past two decades, enormous advances have witnessed in the field of nanogels and their prospective use in controlled-release drug delivery systems and their use in the delivery of imaging agents [23]. Nanogels are usually defined as aqueous dispersions of hydrogel particles [93]. They are categorized into two groups as chemically or physically

cross-linked nanogels. The cross-linking of constituent nanogels by covalent bonds forms the chemically cross-linked nanogels [30], whereas physically cross-linked ones are linked bu non-covalent bonds including hydrophobic interactions, ionic bonds, and hydrogen bonds [25].

Nanogels are very promising as nanocarriers in drug delivery systems due to their high stability, stimuli responsive behavior in response to environmental pH, temperature or ionic strength alterations, and high loading capacity. Because of the ability to load small bioactive molecules within their nanospace, nanogels are unique to carry nanoscale pharmaceuticals [93].

Currently, three approaches have been used for the preparation of nanogels, named as (*i*) template-assisted nanofabrication of nanogels particles; (*ii*) physical self assembly of interactive polymers; (*iii*) polymerization of monomers in micro- or nano-heterogeneous environment, or in homogeneous phase; and (*iv*) chemical cross-linking of preformed polymers [93].

The group of J. Sunamoto described the physical cross-linking and self assembly of cholesterol-modified polysaccharides, such as dextran, pullulan, mannan, and amilopectin, resulting in nanosized hydrogels [24].

Sekine et al. reported the first physically cross-linked nanogel by the assembly of hydrophobically modified polymers in a controlled manner. Self-assembly of hydrophobic cholesteryl and hydrophilic pullulan groups in a dilute aqueous solution resulted in stable nanogels, denominated as cholesterol-bearing pullulan (CHP) nanogels. The cross-linking points are provided by the hydrophobic interaction between hydrophobic cholesteryl groups [24]. CHP nanogels have various advantageous properties, such as their ability to carry bioactive molecules including DNA, RNA, and proteins in their 30 nm diameter space, and their artificial molecular chaperone function [94, 95]. CHP nanogels have been further engineered to develop functional hierarchical nanogels. Hierarchical construction of nanogels is a novel strategy which allows the control of nanostructure, as in the case of the bottom-up assembly of methacryloyl or acryloyl group bearing CHP nanogels [26, 27]. Acryloyl-bearing CHP groups are named as CHPOA nanogels [25]. For instance, their polymerization with PEG containing four-arm terminal thiol groups(pentaerythritol tetra(mercaptoethyl)polyoxyethylene (PEGSH)) by Michael addition formed biodegradable

hybrid hydrogels, with prospective uses in regenerative medicine as scaffold materials for controlled drug/compound release [92, 96].

Although nanogels are beneficial in many terms, nanogel drug delivery systems have several potential drawbacks, such as their low loading capacity for hydrophilic drugs. Sekine et al. included both nanogels and liposomes in their research published in 2012, to utilize the higher hydrophilic drug loading capability of liposomes, and the molecular chaperone-like activity of nanogels to form a stable complex[25]. Thus, nanogel incorporating hybrid are promising candidates as multidrug delivery systems, allowing sequential release from different components of the hybrid system.

2.3 CONTROLLED DELIVERY OF AN ANTICONVULSANT DRUG

Anticonvulsants are pharmaceutical agents used to reduce or prevent the frequency and severity of seizures in various types of epilepsy. They are also used to treat borderline personality disorder and bipolar disorders, due to their mood stabilizer affect. Anticonvulsants are also called as antiepileptic or anti-seizure drugs, because they provide therapeutic effect on symptoms, but not alter the course of epilepsy [28, 29].

Anticonvulsant drugs modulate neuronal excitability by the generation of inhibitory mechanisms [31]. Different types of anticonvulsants have different modes of action, due to the alterations on which receptor in the brain they act on. The two major mechanisms are the blokage of sodium channel activity and enhancement of GABA (γ-aminobutyric acid) action. Other mechanisms in anticonvulsants are the inhibition of glutamate receptors and calcium channels [97].

Second generation anticonvulsants, such as levetiracetam (LEV), gabapentin (GBP), oxcarbazepine (OXC), lamotrigine (LTG), zonisamide (ZNS) and pregabalin (PGB) have been clinically demonstrated to ensure improved performance against drug resistant epilepsy types, with minimal side effects [98]. Pregabalin specifically acts on the central nervous system, prevents the excessive and fast firing of neurons and suppress their spread within the brain during seizures [31].

Controlled delivery of anticonvulsants, specifically PGB has upmost importance for the symptomic treatment of various central nervous system related conditions, such as epilepsy [28, 29], general anxiety disorder (GAD) [30] and neuropathic pain [31]. Epilepsies are a subgroup of brain disorders wherein normal neuronal activity is disrupted, resulting in strange behavior, sensations, or loss of consciousness. Abnormalities in brain wiring or development, and imbalance of neurotransmitters are among the many possible reasons of epilepsy. Along with the anticonvulsant drug therapy which targets seizure control in epileptic patients, either seizure possibility is reduced or epilepsy becomes seizure free [29]. Furthermore, in preclinical studies PGB has been demonstrated to be a great candidate as an analgesic and an active anticonvulsant for epileptic seizures [75]. Generalized anxiety disorder (GAD) is a chronic medical illness characterized by excessive and persistent worry and tension [30]. It approximately has a year prevalence of 3% in the United States [99]. Currently existing treatment for GAD includes physiological treatment together with medicine, such as antidepressants [100]. PGB has been another approved medication option for GAD and it is included in first-line treatment options according to the World Federation of Societies of Biological Psychiatry [101]. Neuropathic pain generally occurs because of a disease affected the neuronal system. The alterations in the structure and function of the neurons result in excessive and rapid excitation of neurons, followed by the neuron damage and eventual pain [31].

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.

The treatment of these three chronic disorders can be addressed by the controlled release of PGB from a stimuli responsive hydrogel system. Firstly, Cevik et al. studied the extended release of PGB from pure pH-sensitive P(MAA-g-EG) hydrogels, and SBS particle containing or SBS disk embedded P(MAA-g-EG) hydrogels. As a result of the drug experiment, conducted at pH 7.4 for 7 h, the percent drug release has elevated values for the pure P(MAA-g-EG) hydrogels up to 86.4 percent [20].

2.3.1 Pregabalin as a Model Drug

PGB is a unique Food and Drug Administration (FDA) approved neuropathic pain medication, specifically for the treatment of diabetic peripheral neuropathy [32]. PGB is a structural alkylated γ-amino-butyric acid, GABA analogue as seen in Figure 2.5, which does not interact with either GABA receptors or GABA itself [97]. GABA is known as the primary suppressive neurotransmitter participates in the central nervous system [102].

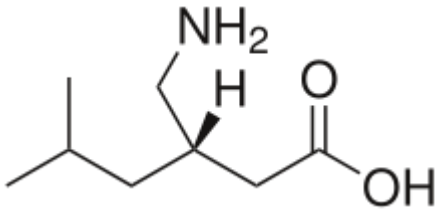

Figure 2.5 The structure of Pregabalin (PGB).

This alkylated GABA analogue binds to the α2-δ protein subunit of the presynaptic neuron's voltage gated calcium channels with high affinity [103, 104]. With this binding, presynaptic calcium channels are closed and calcium influx is decreased at nerve terminals. As a result, excitatory neurotransmitter levels decrease, including glutamate and noradrenaline. Consequently, hyper-excited neurons return to a normal physiologic state in the absence of these excitatory neurotransmitters [102, 103]. In this way, the number of pain signals which are sent by the damaged nerves is decreased. Due to the state dependent activity of PGB, it only regulates the activity of over-excited neurons, and that is why PGB is a unique therapeutic choice for the treatment of epilepsy [105, 106].

PGB has also attracted increasing attention among other second generation anticonvulsants as a result of its extensive use for the treatment of GAD in adults [30]. Despite extensive clinical studies about PGB, controlled and long term controlled release of PGB for inhibition of seizures has not been considered previously [33, 34]. 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.

In clinical studies, single-dose tolerance of PGB is demonstrated to vary between 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 [107]. In addition, usual PGB dosage for adults ranges between 150600 mg/day, and available formulations of PGB capsules are 25, 50, 75, 100, 150, 200, 225 and 300 mg. Preferable dose consideration, on the other hand, is demonstrated as the administration of the drug as 2-3 equally divided doses during the day [108]. Thus, controlled release of PGB is studied with the use of a pH responsive hybrid hydrogel system, wherein the drug will be taken once a day, but release for hours.

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). Eosin Y, L-glutamine (200 mM), penicillin-streptomycin, (stabilized, 10,000 units penicillin and 10 mg streptomycin/mL, pen-strep), trypsin-EDTA (25%), potassium phosphate and ethanol (99.8 %) were obtained from Sigma-Aldrich. Triethanolamine (TEA) and 3,3-dimethylglutaric acid (DMGA) were purchased from Merck (Darmstadt, Germany) and Fisher Scientific (New Jersey, USA), respectively. 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). Sodium chloride was obtained from Merck and, 37% hydrochloric acid and sodium hydroxide was obtained from Riedel-de Haen. The CellTiter-Glo Luminescent Cell Viability Assay was obtained from Promega. 24 well plates and tissue culture dishes were purchased from Greiner Bio-one. PGB was kindly supplied by Abdi İbrahim (Pharmaceutical Company, Istanbul, Turkey), in powder form. CHPOA nanogel was kindly provided by Kyoto University. All chemicals were used without any prior process except MAA which was passed through a column to remove the inhibitor hydroquinone.

The preparation of artificial gastric fluid (AGF, pH 1.2) and artificial intestinal fluid (AIF, pH 6.8) was done as follows. 7 mL of hydrochloric acid (37 wt%) and 2.0 g of sodium chloride were dissolved in 1000 mL of deionized water for the preparation of AGF. To

prepare AIF, potassium dihydrogen phosphate (6.8 g) was dissolved in 500 mL of deionized water, and then pH was adjusted to 6.8. The solution was then diluted to 1000 mL by the addition of deionized water. The preparation of artificial gastric fluid (AGF, pH 1.2) and artificial intestinal fluid (AIF, pH 6.8) was done as follows. The pH adjustment of all buffer solutions was accomplished by the addition of 6 M HCl or NaOH solutions.

3.2 METHODS

3.2.1 Synthesis of Hybrid Hydrogels

3.2.1.1 Bulk synthesis of P(MAA-g-EG) and CHPOA-bearing hybrid hydrogels

For both cases of synthesis under ultraviolet (UV) light and visible light, the P(MAA-g-EG) prepolymer solution consists 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. Separately, 10 mg/mL CHPOA nanogel solution was prepared in 1XPBS and added to the prepolymer mixture in different final concentrations (0, 1.5, and 10 %). Next, the solution consisting the prepolymer solution and the CHPOA nanogel was exposed to UV light (Output wavelength of Ti:sapphire pumped optical parametric amplifier (OPA) was tuned to 300 nm with 44mW power for 20 minutes or exposed to visible light (514 nm and 7.5 mW/cm^2, Coherent Inc., Santa Clara, CA) using argon laser for 20 min. The formed hybrid gels were finally washed by continuous shaking in distilled water for several days at room temperature to remove excess Eosin Y and freeze-dried for 5 hours (Labconco, FreeZone 2.5 liter Benchtop Freeze Dry System).Dry weights were measured.

3.2.1.2 Surface initiated synthesis of CHPOA-bearing P(MAA-g-EG) hybrid hydrogels

For both UV light and visible light induced surface initiated synthesis of hybrid hydrogels, firstly 1 mM Eosin Y solution was applied to the surface of the molds in which the polymer

solution will be poured into and the molds were let to be dried. Afterwards, the prepolymer solution consisting of the monomer mixture including 3.6 g of MAA and 2.0 g of PEGMMA in 50:50 (w/w) ethanol and water solution, together with 0.5 mol % TEGDMA and 225 mM TEA was prepared. Then,10 mg/mL nanogel solution was added into the prepolymer solution at different final concentrations (0, 1, 5, and 10 %) and this mixture was poured into the molds. Then, photopolymerization was performed under UV or visible light with the same conditions applied to the bulk synthesis. Finally, the hybrid gels were washed and freeze-dried under the predefined conditions.

Whole procedure is shown in Figure 3.1.

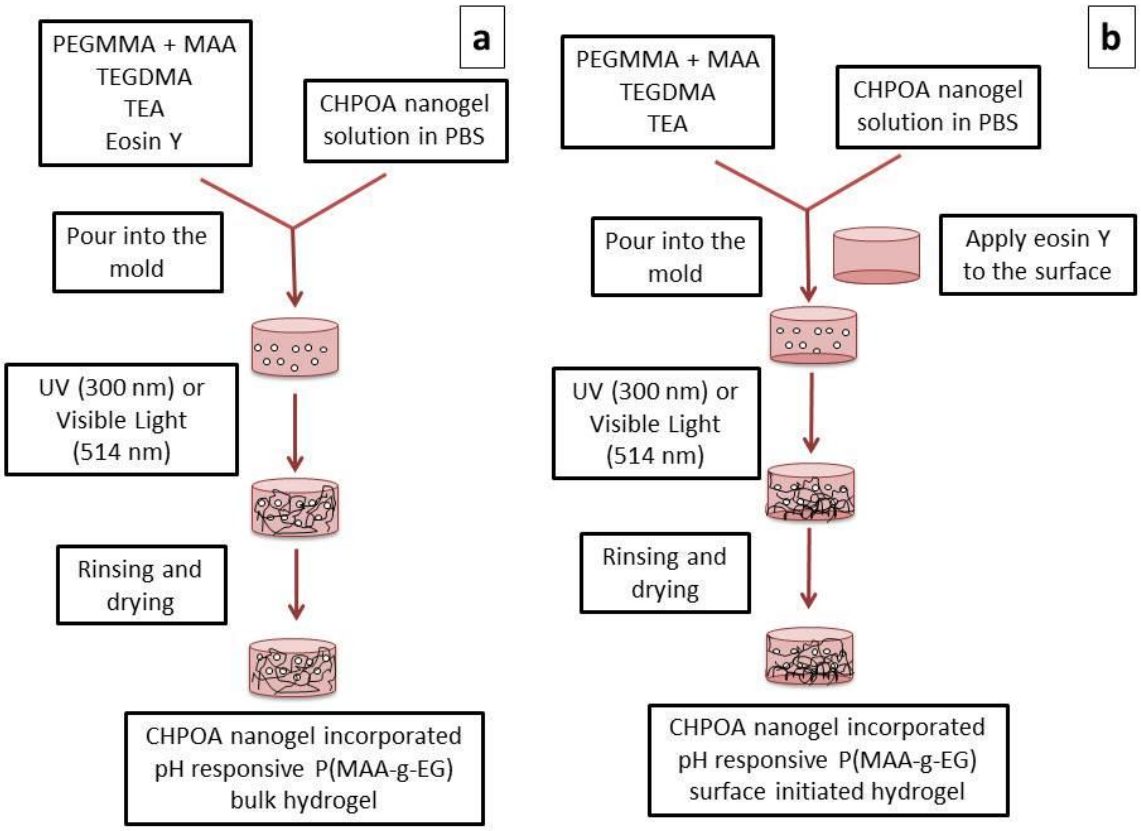

Figure 3.1 Schemeforthe a) bulkand b) surfaceinitiatedpolymerization of hybridpH-responsiveP(MAA-g-EG) hydrogels through UV or visible light photopolymerization.

3.2.2 Characterization of Hybrid Hydrogels

3.2.2.1 Field emission scanning electron microscopy (FE-SEM)

Field emission scanning electron microscope (FE-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 FE-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 Ultra Plus field emission scanning electron microscope. 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 CHPOA nanogel bearing hybrid hydrogels

Dynamic weight swelling experiments were carried out in different pH solutions (pH 2.2, 4.4, 5.5, 6.8, 7.4) to further characterize the pH sensitivity of hybrid hydrogels.

AGF, containing 7 mL HCl (37 wt%) and 2 g NaCl in 1 L of deionized water was used to mimic the stomach environment and this represented the pH 2.2 buffer solution. AIF consisting of 6.8 g KH_2PO_4 in 1 L deionized water was used to mimic the intestine environment [109]. AIF was used as the buffer solutions ranging between 4.4 and 7.4, for dynamic and reversible swelling experiments. The pH adjustment of all buffer solutions was accomplished by the addition of 6 M HCl or NaOH solutions. Each hydrogel was placed into 5 ml of different pH buffers and kept at 25°C. At specified time points, wet weights were measured 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:

$$q = W_s / W_d \quad (1)$$

where Ws is the weight of the swollen hydrogel and Wd is the initial weight of the dried hydrogel.

3.2.3.2 Reversible swelling experiments of P(MAA-g-EG) and CHPOA nanogel bearing hybrid hydrogels

In order to investigate whether CHPOA nanogel-bearing hybrid hydrogels respond to cyclic changes in the pH and to understand the mechanical stability of the gels, the reversible

swelling experiments were performed. Reversible swelling/deswelling behaviors for all types of hydrogels were characterized using pH 2.2 AGF and pH 7.4 AIF prepared as in Section 3.2.1.1 and 3.2.1.2. The main purpose of using this pH range is, to mimic the PGB release profiles 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 [110, 111]. Each hydrogel was placed into 5 ml of different pH buffers and kept at 25°C. At specified time points, wet weights were measured for the calculation of equilibrium weight swelling ratio.

3.2.4 Pregabalin (PGB) Loading

For this study, pregabalin (PGB) was chosen as a model molecule for release studies. PGB stock solution was prepared in 1X 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 µl 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 to determine the concentration of unknown samples.

3.2.5 PGB Release Studies

For PGB release studies, PGB loaded hydrogels were placed in 0.1 M DMGA buffers (The ionic strength of DMGA buffer were maintained with sodium chloride.) at 100 rpm shaker and 25ºC. In order to mimic the stomach environment, the samples were kept in pH 2.2 DMGA buffer for the first 2 hours and for the following 6 hours the PGB release experiment was performed at pH 7.4. mimicking the intestine environment. Every 30 minutes, 500 µl samples were taken and replaced with fresh pH 2.2 (up to 2 hours) and then with pH 7.4

DMGA buffer to sustain sink conditions. PGB concentrations obtained from absorption measurements were used to calculate mass released at time *t* (M_t) using the following equation [112], by performing mass balance:

$$M_t = C_t * V + \Sigma C_{t-1} * V_s \quad (2)$$

Where *Ct* is the concentration of PGB in the release solution at time *t*, *V* is the total volume of release solution (10 ml) and *Vs* is the sample volume that is taken (500 μl). Using the M_t values, the % release of PGB was determined as expressed with the following equation [75]:

$$\% \text{ Mass Released} = (M_t / M_\infty) * 100 \quad (3)$$

where M_∞ (M_{inf}) is the total weight of PGB released during the experiment**.**

3.2.6 Biocompatibility of P(MAA-g-EG) and CHPOA nanogel bearing hybrid hydrogels

3.2.6.1 Culture of HeLa cell line

HeLa cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% Penicillin/Streptomycin. They were grown passages between 8-11. Cells were maintained in a humidified environment at 37°C with 5% CO2.

3.2.6.2 *In vitro* cell viability tests

The P(MAA-g-EG) and CHPOA-bearing hybrid hydrogels were immersed in the complete culture medium for 24 h, at 37ºC before seeding. For static seeding of HeLa cells, all hydrogel samples were located in 24-well plate. Then, 15000 cells/0.2 cm^2 gel surface were seeded onto the gels. After 15 minutes, 1 mL of compete 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 HeLa cells on hydrogels, CellTiter-Glo Luminescent Cell Viability Assay (Promega) was performed for day 1 and 4 to measure intracellular ATP content. Standard curve was generated using ATP solutions at specific concentrations (1, 0.1 and 0.01 μM) prepared in cell culture medium. Next, gels were incubated in 50% CellTiter-Glo reagent and cell culture medium at25ºC, 100 rpm for 15 min. After incubation, 200 μl from each sample was removed and luminescence was measured with a luminometer (Biotek, Synergy H1).

3.2.7 Statistical Analysis

All experiments were performed at least three times. The results were represented as the mean value (±SD, standard deviation) of all samples.

Chapter 4

RESULTS AND DISCUSSION

4.1 CHARACTERIZATION

Pure pH-responsive P(MAA-g-EG) hydrogel together with composite P(MAA-g-EG) hydrogels that include different CHPOA nanogel ratios, as 1% and 5%, were photopolymerized through both UV and visible light exposure. These hybrid hydrogel systems were also synthesized with the use of different polymerization techniques: bulk and surface initiated polymerization methods. For bulk polymerization approach, prepolymer solutions of pH responsive monomers included eosin/TEA photoinitiaton system both for UV and visible light induced photopolymerization. CHPOA nanogels that were obtained from Sasaki et al. were added into the prepolymer solution in different ratios and photopolymerization was carried out subsequently. For surface initiated photopolymerization, the photoinitiator eosin Y was physically adsorbed onto the substrate surface. Thus, photoinitiation reactions and growth of hydrogel network started from the substrate surface. This resulted in a cross-linking gradient within the polymer network. Next, photopolymerization of the pure P(MAA-g-EG) and CHPOA nanogel incorporating P(MAA-g-EG) hydrogel network was carried out via both UV light and visible light exposure. In Table 4.1 synthesized various hydrogel types were categorized and their abbreviations were given in parenthesis.

Table 4.1 Classification and denomination of various gel types.

UV Light Photopolymerization		**Visible Light Photopolymerization**	
Bulk Polymerization	**Surface Initiated Polymerization**	**Bulk Polymerization**	**Surface Initiated Polymerization**
0% nanogel **(B0-UV)**	0% nanogel **(SI0-UV)**	0% nanogel **(B0-Vis)**	0% nanogel **(SI0-Vis)**
1% nanogel **(B1-UV)**	1% nanogel **(SI1-UV)**	1% nanogel **(B1-Vis)**	1% nanogel **(SI1-Vis)**
5% nanogel **(B5-UV)**	5% nanogel **(SI5-UV)**	5% nanogel **(B5-Vis)**	5% nanogel **(SI5-Vis)**

Surface morphology of hybrid hydrogels and the presence of nanogels within the polymer network were analyzed using Field Emission Scanning Electron Microscope (FE-SEM). In addition to FE-SEM characterization of hydrogels, visual examination of different types of hydrogels was shown in Figure 4.1.

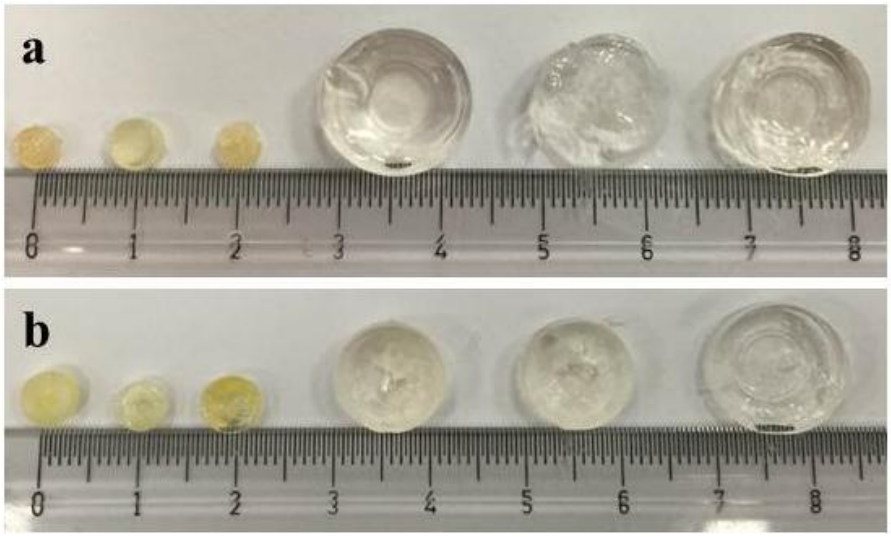

Figure 4.1 Image of visible light photopolymerized pH responsive P(MAA-g-EG) hydrogels in collapsed state (pH 2 DMGA) and in swollen state (pH 7.4 DMGA) as triplets of nanogel free, 1% nanogel incorporating, and 5% nanogel incorporating hydrogels, respectively, for (a) bulk polymerized, and (b) surface initiated polymerized samples.

All hydrogel samples synthesized under visible light, as listed in Table 4.1, demonstrated opaque appearance at low pH (pH 2), while they appeared soft and transparent when at pH 7.4. As was observed from these images, incorporation of nanogels into pH responsive network did not compromise swelling and pH responsive properties, and that hydrogels retained swollen structure at high pH medium, and remained in collapsed state at low pH. In addition, hydrogels in altered groups preserved their integrity despite tenfold increase in size (Figure 4.6) at pH 7.4. Furthermore, as can be observed in both nanogel free and 5% nanogel incorporating hydrogels synthesized through bulk (Figure 4.1a) and surface initiated (Figure 4.1b) polymerization, the sizes of the hydrogels prepared with 5% nanogels at swollen state were larger compared to hydrogels prepared with 1% nanogels.

4.1.1 FE-SEM Characterization of P(MAAg-EG) and CHPOA Nanogel Incorporating Hybrid Hydrogels

The presence of covalently attached nanogels and porous structure of the P(MAA-g-EG) hydrogel were characterized through FE-SEM images.

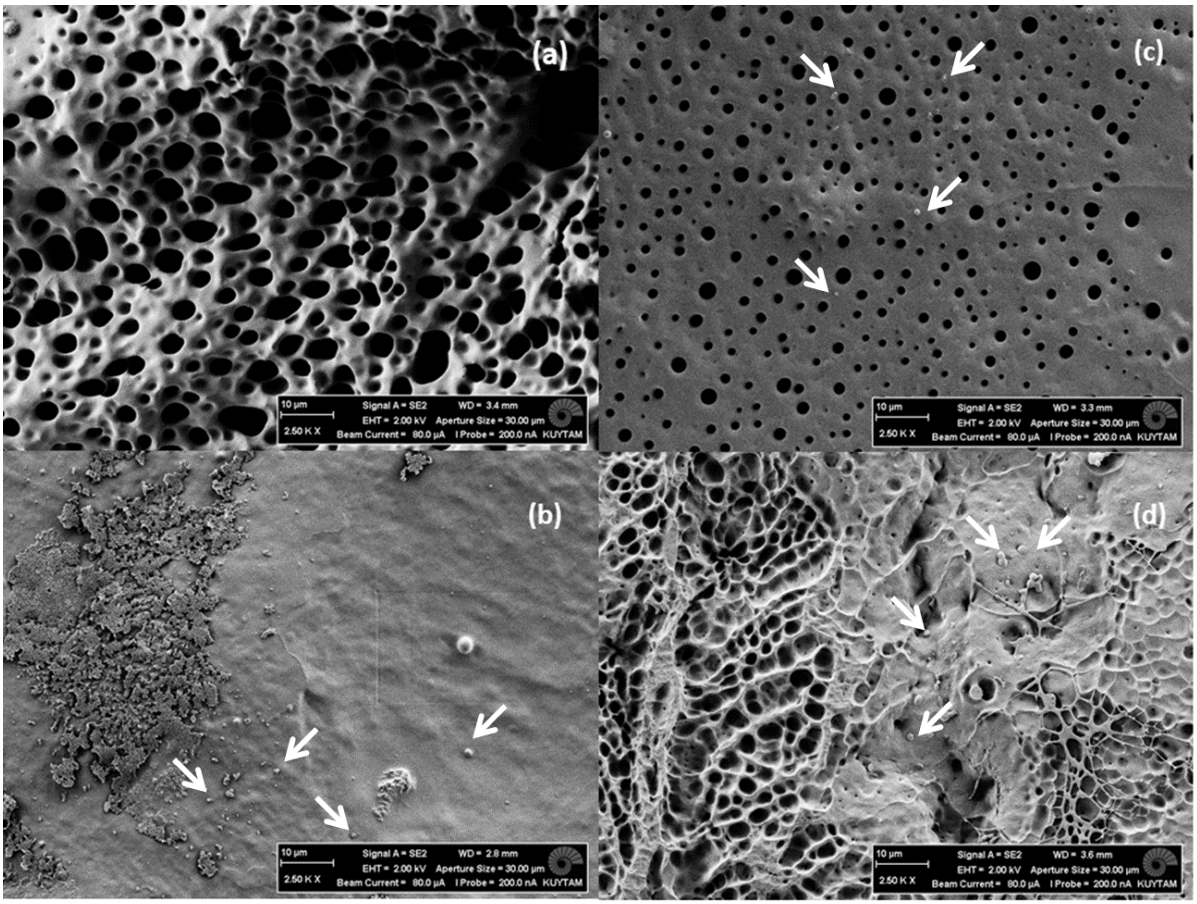

Figure 4.2 Field emission scanning electron microscope images of (a) pure pH responsive P(MAA-g-EG) hydrogel photopolymerized under UV light (b) 1% nanogel incorporating P(MAAg-EG) hydrogel photopolymerized under UV light, (c) 1% nanogel incorporating P(MAAg-EG) hydrogel photopolymerized under visible light, and (d) 5% nanogel incorporating P(MAAg-EG) hydrogel photopolymerized under visible light. (Scale bar: 10 μm).

FE-SEM images demonstrated morphological differences between UV and visible light induced photopolymerized hydrogels,, which is consistent with the previous study[20]. According to the FE-SEM images of bulk polymerized hybrid hydrogels (Figure 4.2), both UV and visible light exposure resulted in the formation of openings on the surface of all hydrogel structures. As can be observed from Figure 4.2a, pore size ranged between 1-10 μm for UV cured pure P(MAA-g-EG) hydrogels, whereas a flat structure was obtained for the hybrid P(MAA-g-EG) hydrogel containing 1% nanogel (Figure 4.2c). As can be observed in both Figure 4.2b (1-3 μm) and Figure 4.2d (2-5 μm), the pore diameter had lower values, compared to the hydrogels photopolymerized with high energy UV light. The dispersed nanogel structures within hydrogel network were demonstrated with white arrows in figures 4.2b through 4.2d. The figures also showed that higher nanogel concentration in the prepolymer solution resulted in higher amount of nanogel domains within pH responsive hydrogel network.

Figure 4.3 demonstrated the hydrogels synthesized by the surface initiated polymerization, where the photoinitiator was physically adsorbed onto a substrate surface. With this approach, photopolymerization started from the substrate-gel interface, and hydrogel growth occurred away from the interface. The left column in Figure 4.3 demonstrates FE-SEM images of the samples at substrate-gel interface of the hydrogels, whereas the right panel demonstrates FE-SEM images of the gel surface for the same sample set. Because of the flat substrate surface, the surface of hydrogels appeared as flat and stiff compared to the gel surface images in Figure 4.3a, c, e, and g. Higher stiffness was observed at substrate-gel interface compared to the stiffness on gel surface for surface initiated photopolymerization. Both the UV cured bare and visible light cured 1% nanogel bearing P(MAA-g-EG) hydrogels had a flat surface structure without any pores, whereas 0.5-4 μm sized pores were observed when nanogel concentration in the prepolymer was increased to 5% for both UV and visible light photopolymerization. Higher pore sizes were also observed on gel surfaces, when 5% nanogel incorporating hydrogels were characterized with FESEM, as can be observed in Figure 4.3d (5-35 μm) and Figure 4h (3-27 μm). These results suggested elevated pore sizes for higher nanogel concentrations within nanogel/hydrogel network. When Figure 4.3e and 4.3f were

compared, rougher surface was obtained at the surface of gel-air interface compared to the surface at substrate-gel interface for 1% nanogel incorporating hybrid hydrogels synthesized via visible light. With the addition of nanogel into the hydrogel network, higher number of nanogels could be observed (Figure 4.3h). Despite higher porosities, better integrities were also observed for hydrogels synthesized via visible light, compared to the ones synthesized with UV light exposure. Better integrities observed via visible light exposure could be related to the efficient photoinitiation and cross-linking reactions taking place in the presence of eosin Y and TEA.

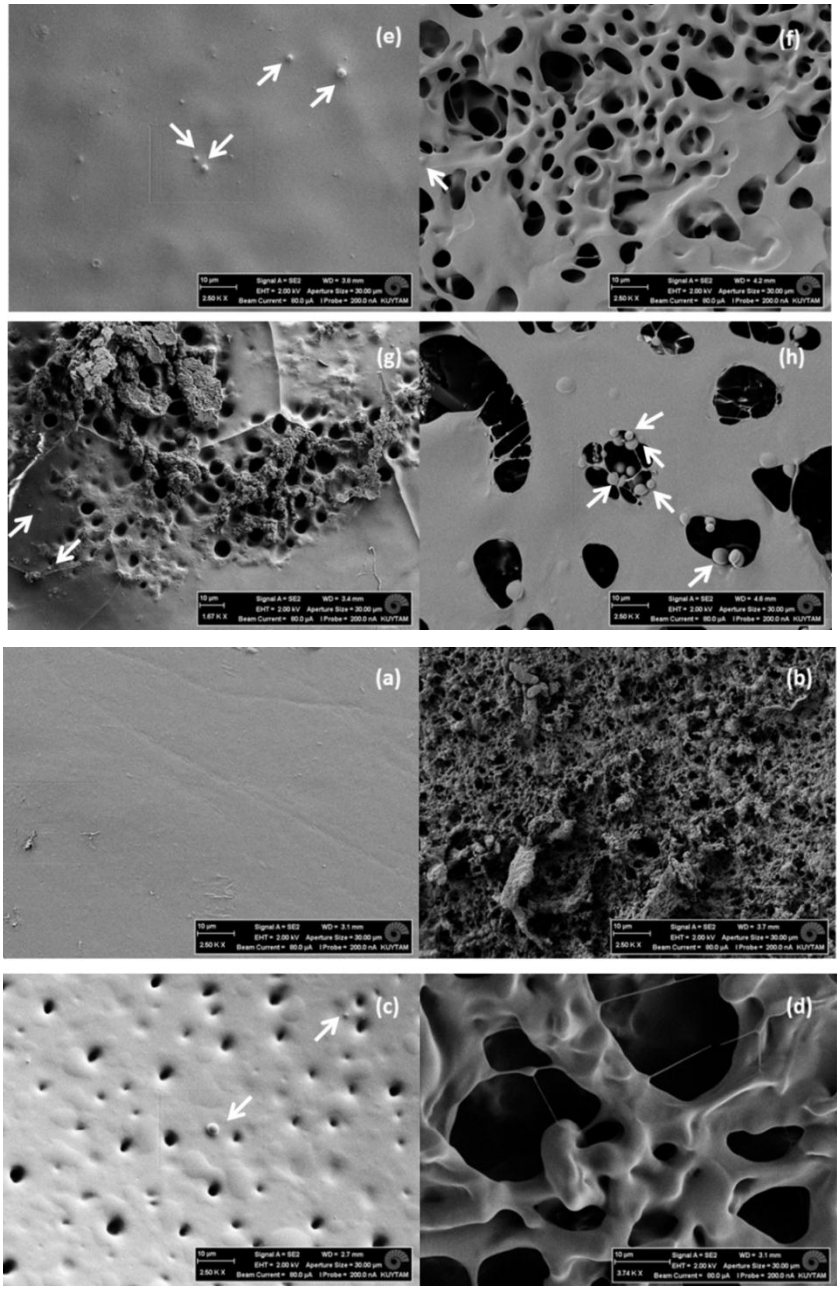

Figure 4.3. Field emission scanning electron microscope images of surface initiated bare pH responsive hydrogel photopolymerized under UV light (a) – substrate-gel interface, (b) – gel surface , 5% nanogel incorporating pH responsive hydrogel photopolymerized under UV light (c) – substrate-gel interface, (d) – gel surface, 1% nanogel incorporating pH responsive hydrogel photopolymerized under visible light (e) – substrate-gel interface, (f) – gel surface, 5% nanogel incorporating pH responsive hydrogel photopolymerized under UV light (g) – substrate-gel interface, and (f) – gel surface (Scale bar: 10 μm).

4.2 SWELLING EXPERIMENTS

4.2.1 Dynamic Swelling Behavior of P(MAAg-EG) and CHPOA Nanogel İncorporating Hybrid Hydrogels

Dynamic swelling experiments were performed to investigate the pH sensitive behavior of the hybrid hydrogels (Figure 4.4). Thus, simulated physiological buffer solutions, named as artificial gastric fluid (AGF), mimicking the stomach environment, and artificial intestinal fluid (AIF), mimicking the small intestine environment, were used with different pH values in swelling studies.

At high pH values, the carboxyl groups in MAA monomer become negatively charged, as a result electrostatic repulsion occurs between neighboring PEG chains. PEG chains within the network act as both hydrogen bonding points with water and also coil extension suppliers. On the contrary, at low pH condition, such as in acidic environment of stomach, the carboxyl groups of MAA become protonated. Hence, in the absence of repulsive forces, hydrogen bonds form between PEG chains and MAA. For the case of anionic P(MAA-g-EG) hydrogels, a collapsed state is retainedat low pH conditions [17, 113]. As shown in Figure 4.4, minimal swelling was observed in acidic condition, where an increased swelling profile was observed with increasing pH in both bulk polymerized and surface initiated polymerized hybrid structures. We also observed maximum swelling at highest pH buffer consistent with previous studies related to similar pH responsive hydrogels [20, 114]. It can also be concluded that

neither photo-polymerization method nor the addition of 5% nanogel adversely influenced pH dependent swelling behavior of CHPOA nanogel incorporating hybrid hydrogels. Furthermore, we obtained a slight increase in the swelling ratio of 5% nanogel incorporating hybrid hydrogels synthesized with surface initiated polymerization (Figure 4.4b) compared to that of bulk polymerized hydrogels (Figure 4.4a). As a separate note, these swelling results could be explained by different pore sizes in 5% nanogel incorporating hybrid hydrogels based on different polymerization approaches. As can be observed in Figure 4.2d, pore size of

5% CHPOA nanogel was significantly lower in bulk polymerized samples (Figure 4.2d) compared to hydrogels formed with surface initiated photopolymerization (Figure 4.3h). Larger pore size in hydrogels synthesized with surface initiated photopolymerization was also supported by FE-SEM images. Critical parameters such as critical volume fraction for hydrogels have been calculated through the use of hydrogel swelling ratios in previous studies. The degree of ionization which is the most effective factor of ionic networks, is related to these critical conditions [115].

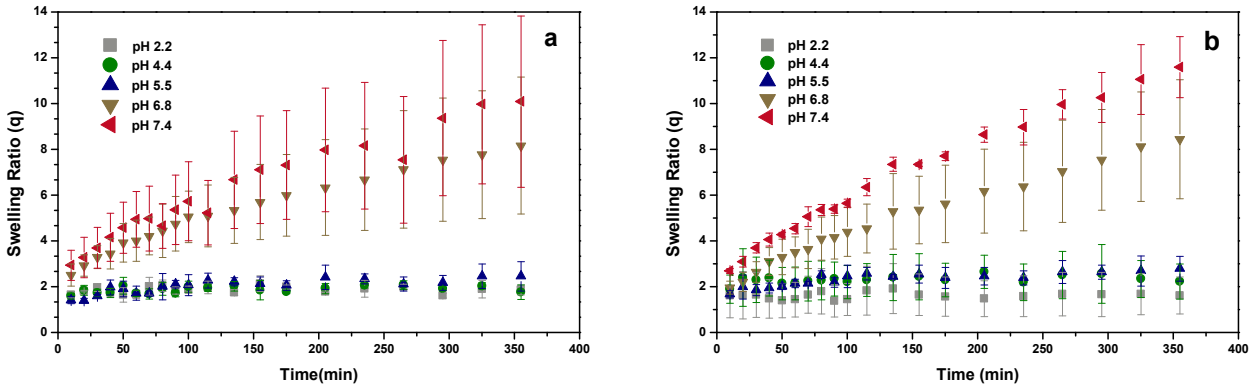

Figure 4.4 Dynamic swelling behavior of 5% nanogel incorporating pH responsive P(MAA-g-EG) hydrogel synthesized by (a) bulk polymerization, and (b) surface initiated polymerization, under visible light.

4.2.2 Reversible Swelling Behavior of P(MAAg-EG) and CHPOA Nanogel Incorporating Hybrid Hydrogels

Reversibility of swelling behavior was examined for composite hydrogels photopolymerized by both UV and visible light, and also by bulk and surface initiated polymerization. For these experiments, hydrogels were incubated at low pH (pH 2 Artificial Gastric Fluid, [109]) and high pH (pH 7.4 Artificial Intestinal Fluid, [109]) cycle, where cyclic behavior with response to differences in environmental pH was investigated (Figure 4.5).

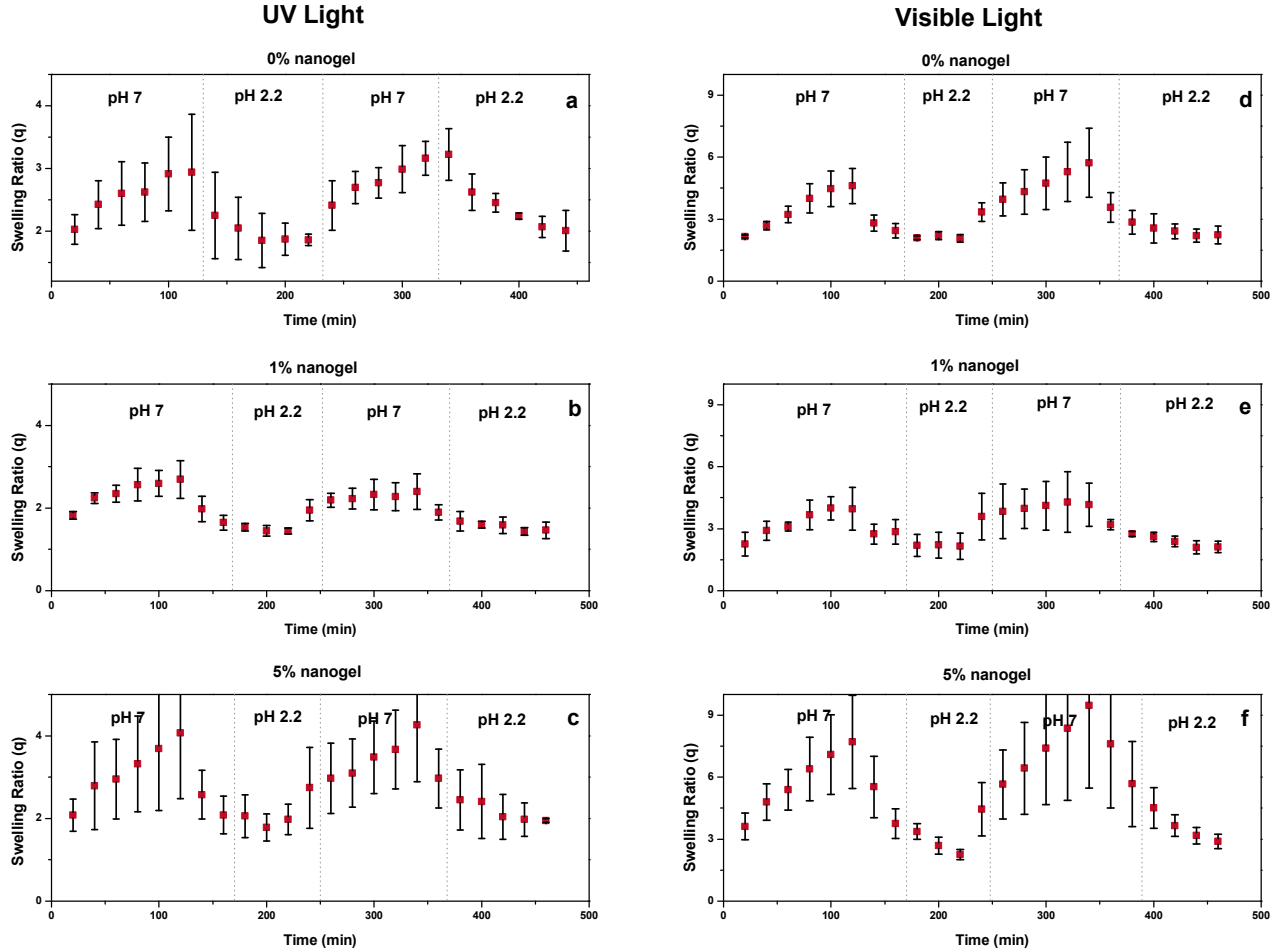

Figure 4.5 Reversible swelling/deswelling behavior of (a) pH responsive P(MAA-g-EG), (b) 1% CHPOA nanogel incorporating P(MAA-g-EG) hydrogel, (c) 5% CHPOA nanogel incorporating P(MAA-g-EG) hydrogel which all had been photopolymerized by UV light. Reversible swelling/deswelling behavior of visible light cured (d) pH responsive P(MAA-g-EG), (e) 1% CHPOA nanogel incorporating P(MAA-g-EG), and (f) 5% CHPOA nanogel incorporating P(MAA-g-EG) hydrogel which all had been photopolymerized by visible light.

Figure 4.5showsthat, hydrogels polymerized under different conditions can respond to altered pH values. With repeated changes in pH, P(MAA-g-EG) hydrogels were observed to swell and collapse repeatedly. Also, it was observed that, the highest swelling could be achieved for bulk polymerized 5% nanogel incorporating P(MAA-g-EG) hydrogels synthesized by visible light exposure, followed by visible light cured bulk polymerized bare P(MAA-g-EG) hydrogels and visible light cured surface initiated polymerized 5% nanogel

incorporating P(MAA-g-EG) hydrogels, respectively (Figure 4.6).Figure 4.6 was obtained using the maximum and minimum swelling ratio values of corresponding hydrogel alterations and conditions. Higher swelling obtained for hydrogels synthesized by bulk polymerization can be explained by uniform distribution of photoinitiator within the prepolymer solution, which resulted in uniform cross-linking network. On the other hand, cross-link density gradients were obtained in hydrogels polymerized by surface initiated polymerization approach, leading to higher stiffness at regions close to substrate-gel interface compared to the cross-link densities at regions close to gel-air interface.

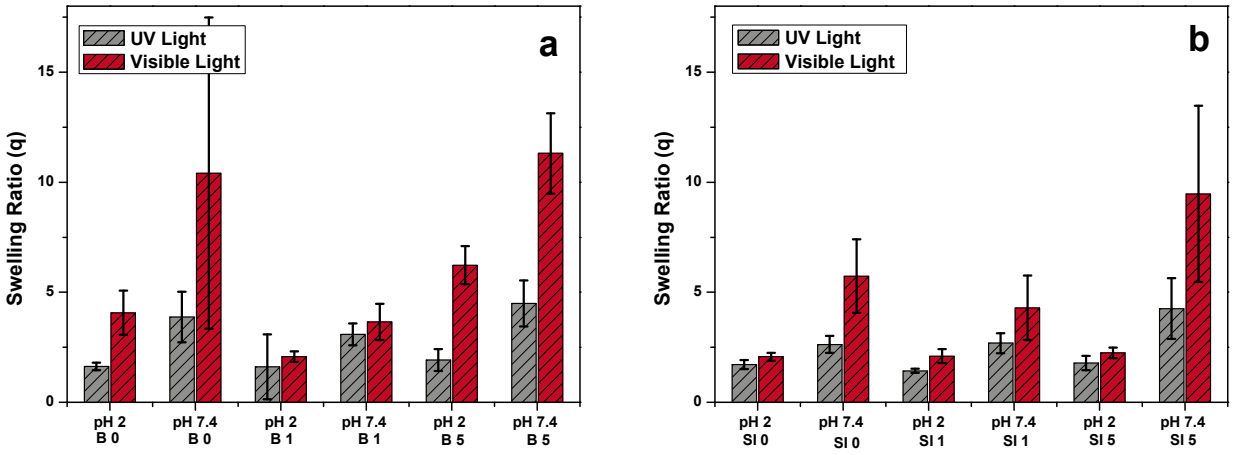

Figure 4.6. Swelling ratio of pH responsive hydrogels synthesized by (a) bulk, and (b) surface initiated polymerization for altered nanogel concentrations (pH 2 and 7.4).

Details of reversible swelling experiments for hybrid hydrogels synthesized by surface initiated polymerization in Figure 4.5, UV cured hydrogels demonstrated lower swelling ratios compared to visible light cured hydrogels. This result was supported by the pore size information obtained from the FE-SEM images in Figure 4.3. The pH responsive P(MAA-gEG) hydrogels synthesized by UV exposure showed smaller pore sizes compared to visible light synthesized hydrogels in zero and 1% nanogel incorporation conditions (Figure 4.3a-4.3f), where the UV cured 5% nanogel incorporating hydrogel (Figure 4.3d) possess bigger pores compared to visible light cured hydrogel (Figure 4.3h). Also, among the reversible swelling results of surface initiated UV cured hydrogels, the swelling ratio of pure pH

responsive P(MAA-g-EG) and 1% CHPOA nanogel incorporating P(MAA-g-EG) hydrogel had lower values compared to swelling ratios of 5% CHPOA nanogel incorporating P(MAA-g-EG) hydrogel, correlated with the pore sizes of these gels.

Incorporation of nanogels into the P(MAA-g-EG) hydrogels influenced weight swelling ratios significantly. As demonstrated in Figure 4.6, for both bulk and surface initiated polymerization samples, and also for both UV and visible light photopolymerization cases, the swelling ratio decreases slightly in 1% nanogel incorporating pH responsive hydrogels compared to the bare P(MAA-g-EG) hydrogels, although a significant increase in swelling ratio was obtained for 5% nanogel incorporating hydrogels. Also, pore diameter of visible light cured 1% nanogel incorporating hybrid hydrogel (1-12 μm) was lower than that of 5% nanogel incorporating hydrogel (Figure 4.3f and Figure 4.3h). These could be explained by the additional bonding formed by the acryloyl groups of acryloyl bearing CHP nanogels, within the hydrogel network, resulting in a stiffer network. It can be concluded that till 1% nanogel concentration, the additional acryloyl groups enhanced the cross-linking events, and the eventual higher stiffness reflected on the lower swelling ratios. However, higher nanogel concentration, 5% in our experiments, compromised cross-linking events and more porous hydrogels resulted in higher swelling ratios. This conclusion was also supported by the hydrogel images as can be seen in Figure 4.1, where larger sized structures were observed for both bulk (Figure 4.1a) and surface initiated (Figure 4.1b) polymerized hydrogels having no or 5% nanogel within their network, compared to the size of 1% nanogel incorporating hydrogels.

4.3 PGB RELEASE STUDIES

Pregabalin release experiments were carried out in DMGA buffer solution at pH 2.0 during the first 2 hours of the experiment, followed by 6 h incubation of the hydrogel samples in DMGA solution at pH 7.4 to mimic the conditions in digestive system. Samples were collected every 30 min, and drug release profiles were measured for altered hydrogel samples [81, 109].

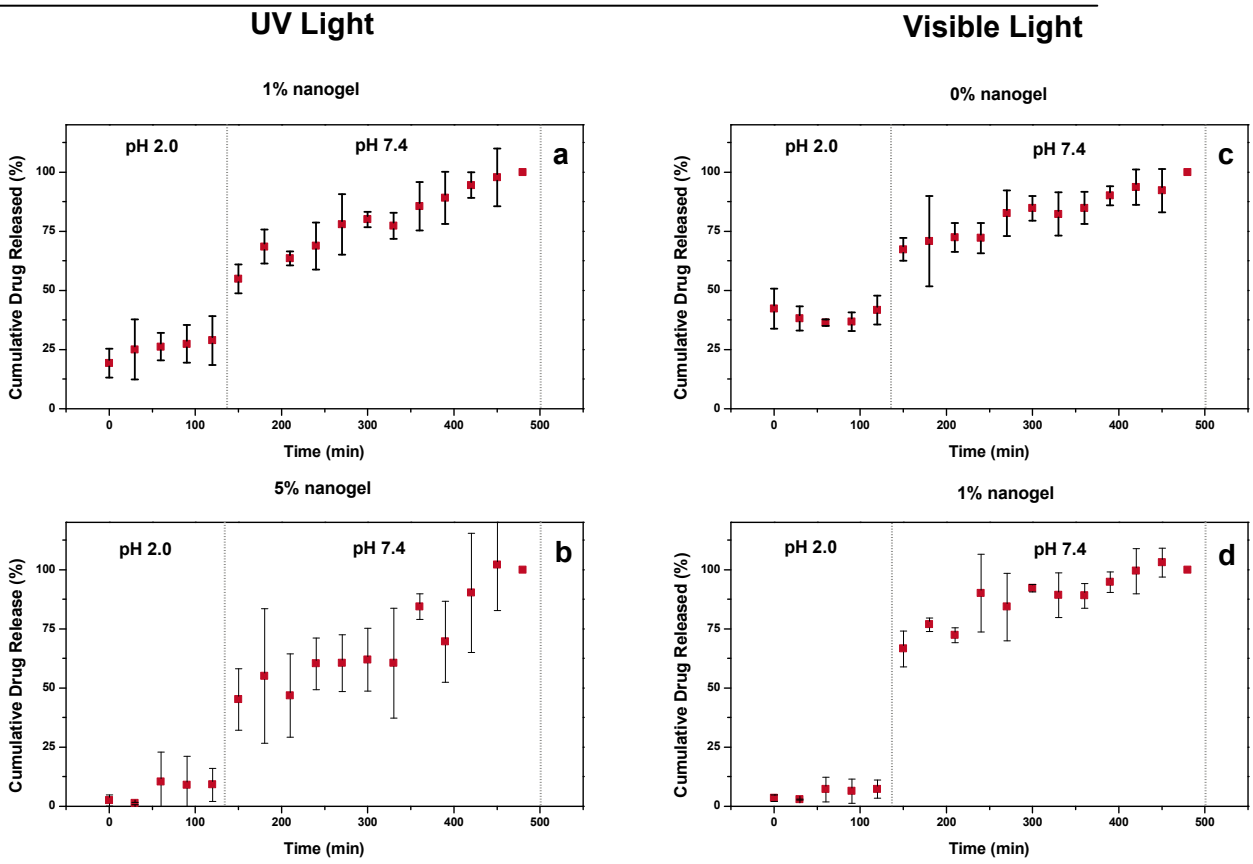

Figure 4.7 Release behavior of PGB at pH 2 (2 h) and pH 7.4 (6 h) from bulk polymerized (a) 1% CHPOA nanogel incorporating P(MAA-g-EG) hydrogel photopolymerized under UV light, (b) 5% CHPOA nanogel incorporating P(MAA-g-EG) hydrogel photopolymerized under UV light, (c) P(MAA-g-EG) hydrogel photopolymerized under visible light, and (d) 1% CHPOA nanogel incorporating P(MAA-g-EG) hydrogel photopolymerized under visible light.

Figure 4.7 shows that PGB can be released at high pH from altered samples synthesized with UV or visible light. Drug release was also observed from all samples independent from the amount of nanogel incorporating within the network. Figure 4.7c demonstrated that at pH 7.4 lower amount of PGB was released from visible light cured bulk polymerized nanogel free hydrogels, as 60.93%. With the addition of CHPOA nanogel in different amounts, percent PGB release increased up to 94.49% (Figure 4.7) at the end of 6 h, as was summarized in Table 4.2. Similar release profile was observed from 5% nanogel incorporating hydrogels synthesized via surface initiated polymerization. On the other hand, only 5.81% (Figure 4.8)

of the PGB released in acidic buffer in UV light cured 5% nanogel incorporating hydrogel (Table 4.2). In addition, PGB release from 1% nanogel incorporating pH sensitive hydrogel synthesized with UV light was lower compared to the hydrogels synthesized with visible light. This result could also be explained by smaller pore size in case of UV polymerization, which was observed in FE-SEM images (Figure 4.2).

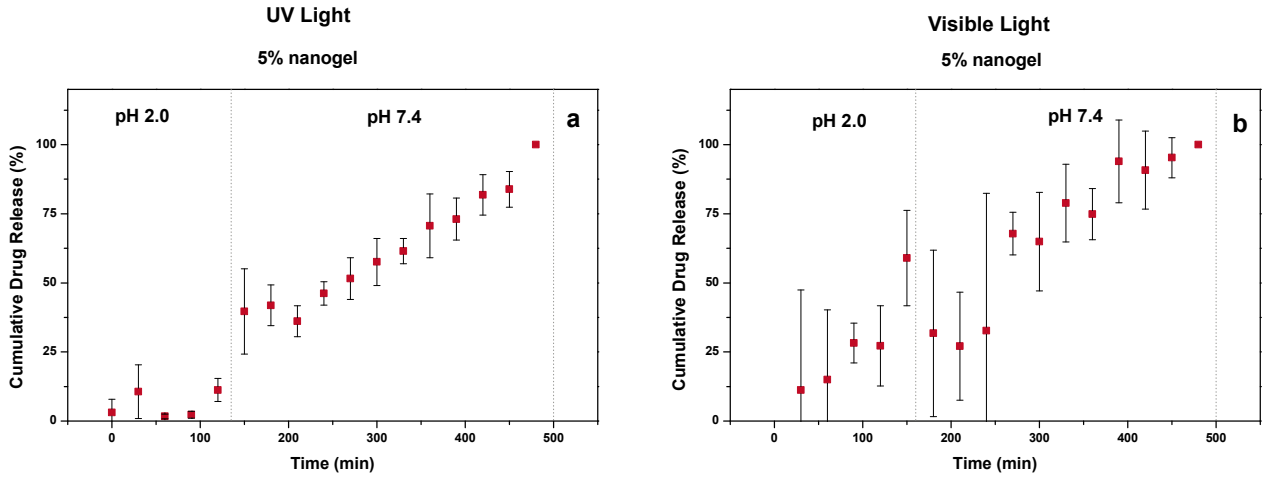

Figure 4.8 Release behavior of PGB at pH 2 (2 h) and pH 7.4 (6 h) from surface initiated polymerized (a) 5% CHPOA nanogel incorporating P(MAA-g-EG) hydrogel photopolymerized under UV light, and (b) 5% CHPOA nanogel incorporating P(MAA-g-EG) hydrogel photopolymerized under visible light.

Above pH 5.4, which is the pK_a value of the MAA [99], P(MAA-g-EG) hydrogels remain in swollen state, and that highest swelling was observed at pH 7.4, as was measured in dynamic swelling experiments (Figure 4.4). These results showed that at low pH environment, for all types of hydrogels minimum amount of PGB release was observed. This observation is also consistent with our previous studies, about the delivery of PGB from pH sensitive P(MAA-g-EG) at acidic pH [20], as well as other studies in the literature about the delivery of various drugs from pH responsive systems [116]. Furthermore, according to the study of Cevik et al., the transport of PGB from both pure P(MAA-g-EG) and SBS incorporating P(MAA-g-EG) hydrogels was fitted to a model to explain the mode of release as swelling-induced or diffusion-induced. It was found that the release of PGB from these systems was swelling controlled diffusion at pH 7.4 [20].

Table 4.2 Comparison of % PGB release for pH responsive hydrogels.

Formulation of Hydrogel	**B1-UV**	**B0-Vis**	**B1-Vis**	**B5-UV**	**SI5-UV**	**SI5-Vis**
PGB Release (%)	74.65	60.93	94.49	93.47	94.19	91.41

Different from the previous in our group, hydrophilic pullulan domains within the nanogel structures were incorporated into pH responsive hydrogels in the present study. These nanogel domains might have contributed to the release of hydrophilic PGB and as a result, PGB delivery from the network is elevated in CHPOA nanogel incorporating hybrid hydrogels [59]. In addition, higher amounts of release of PGB from nanogel incorporating pH sensitive P(MAA-g-EG) hydrogels (Figure 4.7a, 4.7b, 4.7d and Figure 4.8a, and 4.8b) can be explained by high swelling responses for the case of high pH medium in nanogel incorporating networks.

4.4 CHARACTERIZATION OF CELL VIABILITY

Biocompatibility of pH responsive hybrid hydrogels was investigated *in vitro* using HeLa cell line. HeLa cells were seeded onto the surface of pH sensitive hydrogels and HeLa cell viability was characterizedonday 1 and day 4 using cell-titer viability assay. The results of *in vitro* cell viability assay for nanogel free, 1% and 5% nanogel incorporating P(MAA-g-EG) hydrogels synthesized under visible light, both by bulk and surface initiated polymerization, are shown in Figure 4.9. Amounts of ATP measured with cell-titer assay were converted to percent cell survival. According to Figure 4.9, HeLa cell viability was improved on altered hydrogels on day 1 and day 4, and that no toxic effect on cell survival was observed. Also, for both bulk (Figure 4.9a)and surface initiated (Figure 4.9b) polymerizations the addition of CHPOA nanogels into hydrogels improved cell survival. The improved cell survival with the addition of CHPOA nanogels could be explained through better adhesion of HeLa cells on nanogel domains. In addition, higher stiffness obtained with 1% CHPOA nanogel

incorporating hybrid hydrogels compared to 5% CHPOA nanogel incorporating hydrogels might have contributed to the elevated cell survival on day 4 (Figure 4.2d and Figure 4.9).

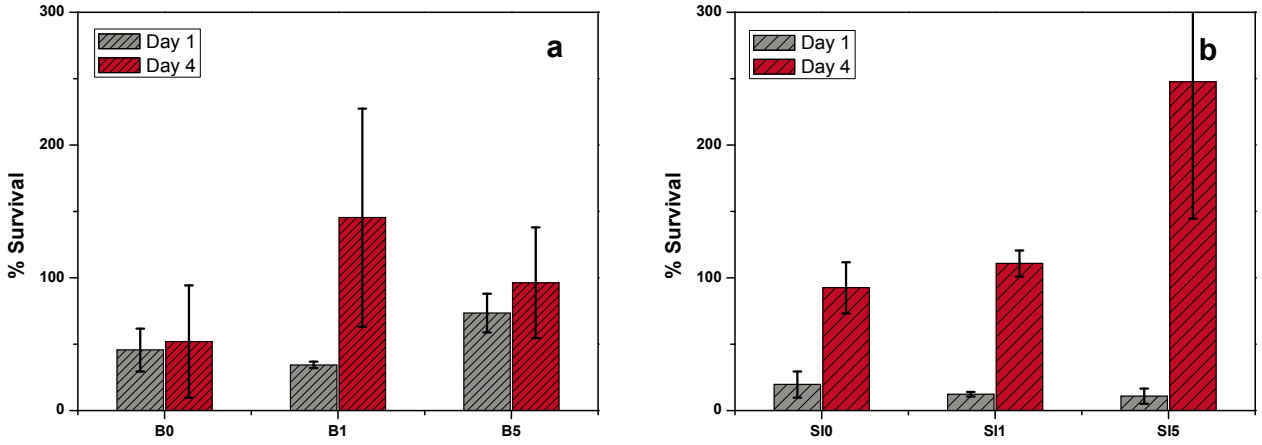

Figure 4.9 Percent survival of HeLa cells on a) bulk, and b) surface initiated polymerized sets of pH responsive P(MAA-g-EG) hydrogels.

CONCLUSIONS

In this study, pH-sensitive hybrid hydrogel systems consisting of pH-responsive poly(methacrylic acid-grafted-ethylene glycol), P(MAA-g-EG), and acryloyl group modified-cholesterol-bearing pullulan (CHPOA) nanogels was developed for controlled delivery of a model drug PGB. For the synthesis of nanogel incorporating stimuli sensitive hydrogels, two different polymerization methods were utilized to synthesize hydrogels: bulk and surface initiated polymerization. Ultra-violet (UV) and visible light-induced approaches were used for bulk polymerization, where photoinitiator was suspended within prepolymer solution. Surface initiated polymerization was used, where photoinitiator was physically adsorbed onto a substrate surface for photoinitiation of polymerization reaction. The presence of covalently attached nanogels and porous structure of the P(MAA-g-EG) hydrogel were characterized by FE-SEM images. pH-sensitive behavior of both the P(MAA-g-EG), and nanogel incorporating P(MAA-g-EG) hydrogels were investigated through dynamic swelling experiments. Reversible swelling experiments with hydrogels demonstrated that hydrogels in altered groups could respond to cyclic pH changes, including hydrogels synthesized with surface initiated polymerization or pure and 1% nanogel incorporating hydrogels. Higher swelling ratios were obtained for the hydrogels synthesized through bulk polymerization and for the 5% nanogel incorporating hydrogels with more porous surface structures. On the other hand, swelling ratio of hydrogels incorporating1% nanogel, had higher values due to additional acryloyl groups, which might have contributed to cross-linking events and improved stiffness of hydrogel network. Moreover, the release of pregabalin from various types of hydrogels could be tuned with variation in environmental pH. Finally, *in vitro* viability results for all hydrogels in altered groups, including the UV or visible light photopolymerized pure P(MAA-g-EG) and nanogel-incorporating hydrogels, synthesized through bulk or surface initiated polymerization approaches, were found as nontoxic, as was observed in cell survival assay. In addition to pH-responsive characteristics, the hydrophilic

P(MAA-g-EG) hydrogel system can be utilized as a vehicle for specific hydrophilic molecules within their structure. On the other hand, the CHPOA nanogels have affinity towards hydrophobic substances, such as DNA, proteins, and various drugs. Thus, these new hybrid hydrogels are promising reservoirs for sequential release of multiple molecules with altered hydrophobicities.

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VITA

Günce Ezgi Cinay was born in İstanbul, Turkey in 1990. She graduated from Hüseyin Avni Sözen Anatolian High School in 2008. Same year, she started Chemistry program in Istanbul Technical University and afterwards to Molecular Biology and Genetics program in 2010. Günce received her double major Bachelor of Science degree in 2013. Then she started her graduate school education at Chemical and Biological Engineering at Koc University in 2013. She worked in a project on the "Nanogel-incorporating pH Responsive Composite Hydrogels for Controlled Drug Delivery" supervised by Professor Seda Kızılel. Her current interest is related to the delivery of therapeutic agents from stimuli sensitive hydrogel systems for biomedical engineering