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Research Article

Comparison of Crosslinker Types and Initiation Systems of Thermoresponsive PNIPAM Hydrogels

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ABSTRACT

Thermoresponsive hydrogels are three-dimensional polymer networks which undergo conformational changes in aqueous media depending on the external temperature. As the lower critical temperature (LCST) is close to the body temperature, poly(N-isopropylacrylamide) (PNIPAM) is the main thermoresponsive hydrogel used for biomedical applications. Below LCST, PNIPAM hydrogels swell in aqueous media, above LCST they become insoluble and shrink. This behavior makes it possible to design drug release systems controlled by external temperature. Swelling/shrinking response of PNIPAM hydrogel depends on several factors such as crosslinker type, crosslinking density, hydrophobic/hydrophilic balance and initiator type. In this study, the effects of the initiation system and the crosslinker type on different thermoresponsive hydrogels were compared. For this purpose, thermoresponsive hydrogels were synthesized by using ethylene glycol dimethylacrylate (EGDMA) and N,N′-ethylene bisacrylamide (EBAM) as crosslinkers via photo and thermal initiation systems. The hydrogels were characterized by scanning electron microscope (SEM) and FTIR spectroscopy. Effects of the initiation system and the crosslinker type on the release, swelling behavior, morphology and the biocompatibility behavior of the hydrogels were investigated. The hydrogels synthesized with EBAM demonstrated more promising results compared to the one synthesized EGDMA. It was concluded that poly(EBAM-co-NIPAM)-P has the highest swelling ratio and poly(EBAM-co-NIPAM)-T is the most biocompatible hydrogel. In terms of release characteristics, there was not a significant difference between the hydrogels, even though their swelling characteristics differ.

Keywords: *Thermoresponsive hydrogels, photopolymerization, thermal polymerization*

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Sıcaklığa Duyarlı PNIPAM Hidrojellerde Çapraz Bağlayıcı ve Başlatıcı Sisteminin Karşılaştırılması

ÖZET

Sıcaklığa duyarlı hidrojeller, dış ortamın sıcaklığına bağlı olarak sulu ortamda yapısal değişikliklere uğrayan üç boyutlu polimer ağ yapılardır. Düşük kritik sıcaklık (LCST) vücut sıcaklığına yakın olduğu için, poli (N-izopropilakrilamid) (PNIPAM), biyomedikal uygulamalar için en yaygın olarak kullanılan sıcaklığa duyarlı hidrojeldir. LCST'nin altında, PNIPAM hidrojelleri sulu ortamda şişer, LCST'nin üzerinde çözünmez hale gelir ve büzülürler. Bu davranış, dış sıcaklık tarafından kontrol edilen ilaç salım sistemlerinin tasarlanmasını mümkün kılar. PNIPAM hidrojelinin şişme/büzülme tepkisi, çapraz bağlayıcı tipi, çapraz bağlanma yoğunluğu, hidrofobik/hidrofilik denge ve başlatıcı tipi gibi çeşitli faktörlere bağlıdır. Bu çalışmada, farklı başlatıcı sistemlerinin ve çapraz bağlayıcıların sıcaklığa duyarlı hidrojeller üzerindeki etkileri karşılaştırılmıştır. Bu amaçla, foto ve termal başlatıcı sistemleri aracılığıyla çapraz bağlayıcı olarak etilen glikol dimetilasrilat (EGDMA) ve N, N′-etilenebis akrilamid (EBAM) kullanılarak sıcaklığa duyarlı hidrojeller sentezlenmiştir. Hidrojeller FTIR spektroskopisi ve taramalı elektron mikroskobu ile karakterize edilmiştir. Başlatıcı sisteminin ve çapraz bağlayıcı tipinin salım, şişme davranışı, morfoloji ve hidrojellerin biyouyumluluk davranışı üzerindeki etkileri incelenmiştir. EBAM ile sentezlenen hidrojeller, EGDMA ile sentezlenenlere kıyasla daha uyumlu sonuçlar göstermiştir. Poli(EBAM-ko-NIPAM)-P'nin en yüksek şişme oranına sahip olduğu ve poli(EBAM-ko-NIPAM)-T'nin en biyouyumlu hidrojel olduğu sonucuna varılmıştır. Salım davranışları açısından, şişme özellikleri farklı olsa bile hidrojeller arasında önemli bir fark yoktur.

Anahtar Kelimeler: *Sıcaklığa duyarlı hidrojeller, fotopolimerizasyon, termal polimerizasyon*

I. INTRODUCTION

Hydrogels are three-dimensional hydrophilic polymer networks which swell in the presence of water or physiological fluids. Their water-absorbing property and their similarity to living tissue brings many opportunities for applications in biomedical areas such as fabrication of contact lenses, drug delivery/release systems, tissue engineering scaffolds and wound dressings [1]. Stimuli responsive hydrogels are hydrogels which undergo reversible conformational changes in aqueous media depending on the external stimuli such as temperature, pH, light, ionic strength, specific molecule/ligand [2]. Among the stimuli responsive hydrogels, thermoresponsive hydrogels attract an increasing interest for biomedical applications as their conformational changes can be triggered by ambient and physiological temperatures [3]. One of the most common thermoresponsive hydrogels is poly(N-isopropylacrylamide) (PNIPAM) hydrogel as the phase transition temperature is close to the body temperature [4]. Below the lower critical temperature (LCST), the temperature at which the phase transition occurs, PNIPAM hydrogels form hydrogen bonding causing swelling in water, whereas as the temperature increases hydrogen bonds are broken, thus the hydrogels shrink. This behavior makes it possible to design drug release systems controlled by external temperature [5, 6].

Swelling/shrinking response of PNIPAM hydrogel depends on several factors such as crosslinker type, crosslinking density, hydrophobic/hydrophilic balance and initiator type [7]. There are several studies in literature reporting the effect of crosslinking on the characteristics of hydrogels [8]. Gökçeören et. al. reported a comprehensive study of the effect of crosslinker/monomer ratio and crosslinker structure on thermodynamic properties and network parameters of PNIPAM hydrogels [7]. They have mainly discussed the thermodynamic and mechanical properties of tetraallylammonium bromide-cross-linked NIPAM hydrogels. In another study, researchers reported an investigation of the effect of crosslinking agents on the swelling capacities of hydrogels [9]. They evaluated the swelling capacity of hydrogels by using four different crosslinkers; ethylene glycol dimethacrylate (EGDMA), butanediol diacrylate (BDDA), N,N'-methylenebis(acrylamide) (MBA), and trimethylolpropane triacrylate (TMPTA) in their synthesis. The hydrogels showed a decreasing swelling capacity with increasing concentration of crosslinkers, and EGDMA hydrogels showed the highest swelling among the others. Coughlan demonstrated that the crosslinking concentration of the hydrogel and the nature of the loaded drug are important in thermal control of drug release from PNIPAM hydrogel [10]. Previously, in our group, a plant-oil based crosslinker was introduced to the synthesis of thermoresponsive hydrogels and the effect of crosslinker/monomer ratio on hydrogel characteristics was investigated [5]. Despite the studies regarding the crosslinker effect on hydrogel characteristics reported in literature, only a few studies have been published on the effect of initiator on hydrogels. Xiao showed that with increasing initiator concentration, faster shrinking/swelling dynamic response was obtained. However only one initiation system, TEMED/APS redox couple, was investigated [4]. In another study, a comparative study of two initiation systems was reported in terms of structure and properties of the cross-linked polyacrylic acid hydrogels [11]. They concluded that with the ammonium persulfates sodium sulfite Mohr's salt initiating system, higher degrees of swelling in water are attained as compared to the gels prepared with the TEMED/APS initiating system.

The purpose of the study is to synthesize thermoresponsive hydrogels by using a hydrophobic crosslinker (EGDMA) and a hydrophilic crosslinker (EBAM) via photo and thermal initiation systems, and to compare the effects of these two parameters on the release, swelling behavior, morphology and biocompatibility of the hydrogels. To the best of our knowledge, this is the first example of the comparison of crosslinker type and initiation system of thermoresponsive hydrogels simultaneously in terms of release, swelling behavior, morphology and biocompatibility.

II. EXPERIMENTAL

A. MATERIALS

Ethylene glycol dimethyl acrylate (EGDMA), N,N,N′,N′-tetramethylethylene diamine (TEMED), N-Isopropylacrylamide (NIPAM), N,N'-ethylene bisacrylamide (EBAM), potassium persulfate (KPS), 2,2-dimethoxy-2-phenylacetophenone (DMPA), quercetin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoline bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM) were supplied from Sigma Aldrich. L-glutamine (Gibco) was supplied from Thermo Fisher Scientific. Fetal bovine serum (FBS) was supplied from Lonza. Solvents were supplied from Merck. Distilled water was obtained by using ELGA Purelab Option device. PhotoLab 6600 UV-VIS Spectrophotometer was used for UV absorbance measurements. Eliza Plate Reader (AMR-100 Micro plate) was used to perform cell viability study. MMM Vacucell vacuum oven was used to perform thermal polymerization and to dry

hydrogels. Photopolymerization reactions were performed under UV light, UVGL-58 230V, 50Hz lamp at 365 nm wave length. During the release experiments, 10000 MW dialysis membranes were used. IR characterization of compounds and polymers were performed by using JASCO FT/IR-6000 Spectrometer. SEM imaging of the hydrogels were performed by FEI QUANTA 450 FEG ESEM scanning electron microscope.

B. SYNTHESIS OF THERMORESPONSIVE HYDROGELS

Two initiation systems were used with two different types of crosslinkers to synthesize the thermoresponsive hydrogels, so four different hydrogel samples were prepared and characterized.

Photopolymerization

Poly(EBAM-co-NIPAM)-P and poly(EGDMA-co-NIPAM)-P were synthesized by photopolymerization. For both hydrogels, NIPAM (2 mmol) was used as the thermoresponsive monomer and DMPA (0.01 mmol) as the initiator. EBAM (0.02 mmol) and EGDMA (0.02 mmol) were used as the crosslinkers to synthesize poly(EBAM-co-NIPAM)-P and poly(EGDMA-co-NIPAM)-P, respectively. All of the polymerization reactions were performed in 1 mL distilled water under UV irradiation at 365 nm for 10 min. at 25°C [12]. After the polymerization, the hydrogels were washed with water and methanol to remove unreacted monomers and kept in vacuum oven at 25°C for 24 hours.

Thermal polymerization

Poly(EBAM-co-NIPAM)-T and poly(EGDMA-co-NIPAM)-T were synthesized by thermal polymerization. For both hydrogels, NIPAM (2 mmol) was used as the thermoresponsive monomer and TEMED/KPS (0.01 mmol) redox initiation system as the initiator. EBAM (0.02 mmol) and EGDMA (0.02 mmol) were used as the crosslinkers to synthesize Poly(EBAM-co-NIPAM)-T and poly(EGDMA-co-NIPAM)-T, respectively. All of the polymerization reactions were performed in 1 mL distilled water [12]. Polymerization was carried out at 40°C in the oven overnight. After the polymerization, the hydrogels were washed with water and methanol to remove unreacted monomers and kept in vacuum oven at 25°C for 24 hours.

C. MEASUREMENT OF SWELLING RATIO

For the swelling ratio (SR) study, the hydrogels were swollen in distilled water at 25°C. The gravimetric method was conducted to calculate the swelling ratio of the hydrogels. After placing hydrogels into 1 mL distilled water, they were taken at certain time intervals and weighed. The swelling ratio of the thermoresponsive hydrogels was determined as below:

$$SR = \frac{Ww - Wd}{Wd} \times 100 \quad (1)$$

Ww: weight of swollen hydrogel,
Wd: weight of dry hydrogel.

D. IN VITRO RELEASE PROFILES

After that 5 mg of hydrogel was placed in dialysis membrane with 2 mL of distilled water, the membrane was put into 10 mL of 100 μM quercetin solution. The solution was kept for 12 hours at room temperature for loading. Hydrogels were measured twice before and after loading by UV/Vis spectrophotometer. Differences between the measurements indicate the loaded quercetin to hydrogels in percentages. Release experiments were carried out at 25°C and body temperature (37°C) separately but simultaneously. After the dialysis membrane was put into 10 mL of pH 7.4 buffer solution, UV absorbance at 371 nm of buffer solution was monitored by taking samples at certain time intervals.

E. IN VITRO CYTOTOXICITY ASSAYS

Cytotoxicity experiments were carried out by using COS-7 cells (African green monkey kidney fibroblast-like cell line). The cells were cultured in a complete culture medium composed of Dulbecco's Modified Eagle Medium (DMEM), high glucose with %10 L-glutamine (Gibco) and supplemented with 10% fetal bovine serum (FBS, Lonza®). COS-7 cells were seeded in 24-well cell culture plates (at 14×10^4 cells in 500 μL of medium per well), incubated at 37°C in 5% CO_2 for 24 h to allow attachment and then the 250 μL of the medium was replaced with 250 μL of different concentrations monomers and polymers in PBS or as a control group with only PBS or as a negative control with DMSO. All experiments were carried out in duplicates. Cytotoxicity was measured after a further 24 h of incubation.

The viability of cells was assessed using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [13]. The MTT cytotoxicity test depends on the enzymatic activity of metabolically active living cells to convert yellow tetrazolium salt into insoluble purple formazan. The intensity of formazan formed is evaluated by colorimetric measurements and directly proportional to the amount of living cells.

To carry out MTT assay, 100 μL of sterilized MTT (5 mg/mL) reagent was added to each well and plates were incubated at 37°C in 5% CO_2 for 4h. Then, the MTT solution was discarded and 1 mL of DMSO was added to each well to dissolve formazan crystals. After cell plate was incubated in the dark for 15 min. on an orbital shaker, 100 μL of the dissolved formazan was added into 96-well plate to measure the absorbance at 570 nm using an Eliza Plate Reade. Cell viability was calculated according to the following formula:

$$\text{Cell Viability (\%)} = \frac{\text{Absorbance of the test sample}}{\text{Absorbance of control group}} \text{ x } 100 \qquad (2)$$

III. RESULTS AND DISCUSSION

In this study, four different thermoresponsive hydrogels were produced by using EBAM and EGDMA as crosslinkers via photo and thermal initiation systems. EBAM based hydrogels synthesized by photopolymerization and thermal polymerization are defined as poly(EBAM-co-NIPAM)-P and poly(EBAM-co-NIPAM)-T, respectively. EGDMA based hydrogels are defined as poly(EGDMA-co-NIPAM)-P and poly(EGDMA-co-NIPAM)-T synthesized by photopolymerization and by thermal

polymerization, respectively. NIPAM was used as the thermoresponsive monomers for all hydrogel samples. Two initiation systems were used to prepare the hydrogels, one is photopolymerization with DMPA and the other one is thermal polymerization with TEMED/KPS redox couple. The yield was obtained higher than 95% for all the hydrogels.

A. CHARACTERIZATION OF THERMORESPONSIVE HYDROGELS

FTIR spectra of the hydrogels are shown in Figure 1. As the hydrogels are mainly composed of NIPAM, they all have almost identical spectra. Since 1% of crosslinker ratio is quite low, the functional groups arise from the crosslinker cannot be seen at the FTIR spectra. The FTIR bands at 3429 cm^{-1}, 3277 cm^{-1}, 3017 cm^{-1} represent NH stretching and the bands at 2971 cm^{-1}, and 2925 cm^{-1} represent vibrations of $-CH_3$ of the side chains of PNIPAM, $-CH_2$ of the main chains, respectively. The bands at 1636 cm^{-1}, and 1539 cm^{-1} represent C=O (Amide I) and N-H (Amide II) stretching, respectively.

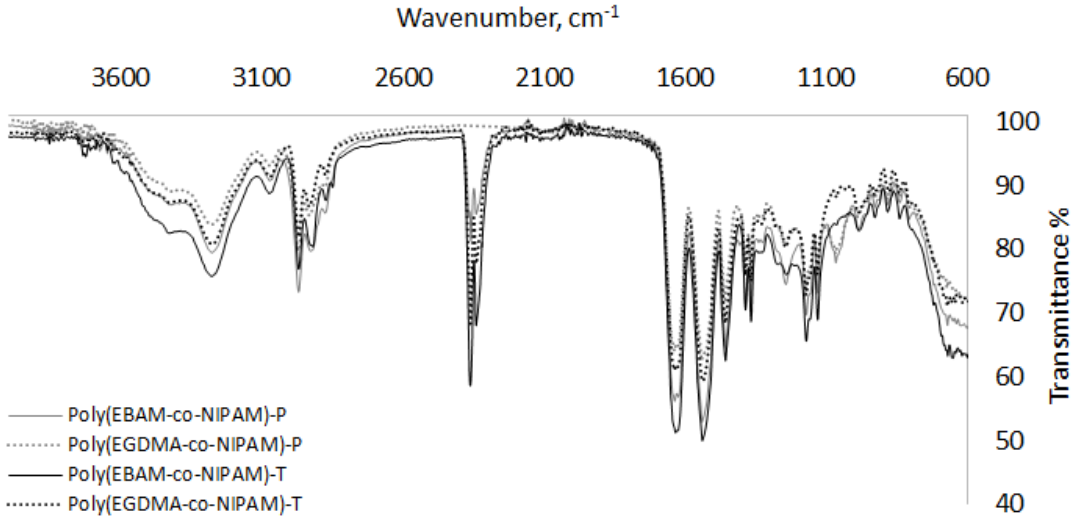

Figure 1. *FTIR Spectra of the hydrogels.*

B. SWELLING BEHAVIORS AND RELEASE PROFILES

Following the characterization of hydrogels, swelling characteristics were investigated. Figure 2a shows that all hydrogel samples are swelling in water over time. The difference in the appearance of dry and wet hydrogel can be seen evidently after introducing water to the hydrogel. The hydrogels synthesized with EBAM demonstrated higher swelling ratio than those synthesized with EGDMA. This can be explained due to hydrophilic character of EBAM. As EBAM hydrogels are cross-linked hydrophilic polymer networks, they tend to swell easily and provide high water content in the network. Figure 2b shows the comparison of swelling behaviors of poly(EBAM-co-NIPAM)-P and poly(EBAM-co-NIPAM)-T hydrogels. Between these two, poly(EBAM-co-NIPAM)-P has the highest swelling ratio. The difference between photo- and thermal polymerization was the reaction time. Poly(EBAM-co-NIPAM)-P was polymerized within 10 min., whereas poly(EBAM-co-NIPAM)-T was synthesized by overnight thermal polymerization. The polymerization time may have an effect on the

network structure, particularly on the softness of the network. Fast reaction resulted in lower degree of crosslinking and a softer hydrogel, so that poly(EBAM-co-NIPAM)-P absorb water faster than poly(EBAM-co-NIPAM)-T. This phenomenon can be confirmed by SEM images (Figure 4). Additionally, hydrogels synthesized with EGDMA were decomposed after 1 hour, which was the reason why Figure 2a showed the swelling ratio results until 60 min.

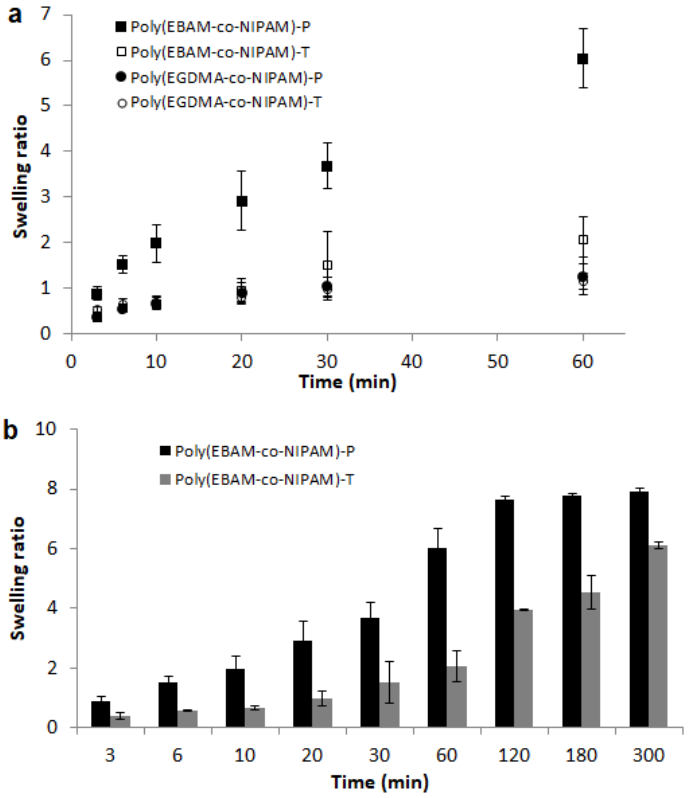

Figure 2. *Swelling behavior of hydrogels;* ***(a)*** *60 min. profiles of all of the hydrogels and* ***(b)*** *5h profiles of poly(EBAM-co-NIPAM)-T and poly(EBAM-co-NIPAM)-P.*

In order to evaluate the effect of crosslinker and initiation system on loading and release behaviors of hydrogels, quercetin was used as a model drug molecule [5]. Quercetin loading processes were carried out for all of the hydrogels separately and release behaviors were investigated individually at room temperature (25°C) and body temperature (37°C). The release profiles are given in Figure 3. As shown in the Figure 3, standard deviations of the release results of EGDMA hydrogels are higher than the standard deviations of release results of EBAM hydrogels. This situation may be explained due to the unstable network of EGDMA hydrogels. As they were decomposed over time, it was hard to control the release, whereas EBAM hydrogels have consistent release results thanks to their stable cross-linked networks. Upon evaluation of overall drug release profiles, it was concluded that there is not a significant difference between the hydrogels, even though their swelling characteristics differ.

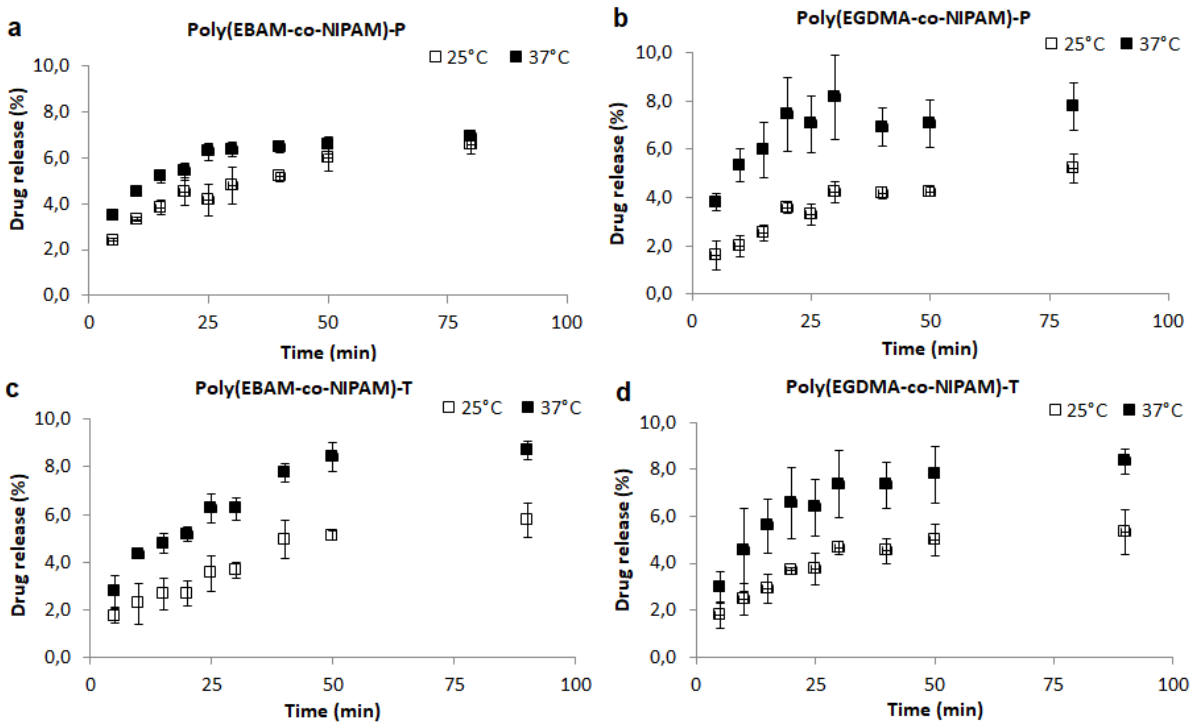

Figure 3. *Release profiles of* ***(a)*** *poly(EBAM-co-NIPAM)-P;* ***(b)*** *poly(EGDMA-co-NIPAM)-P;* ***(c)*** *poly(EBAM-co-NIPAM)-T;* ***(d)*** *poly(EGDMA-co-NIPAM)-T 25°C and 37°C.*

C. MORPHOLOGY OF HYDROGELS

Figure 4 shows SEM images of the hydrogels. These images demonstrate that the hydrogels synthesized by using the photoinitiator have spongy structures compared to the hydrogels synthesized by using the thermal initiator, which have solid texture. We believe that the photopolymerization resulted in less cross-linked network that can be confirmed by swelling behaviours (Figure 2b). Among the photo-polymerized hydrogels, the one synthesized with hydrophilic crosslinker (poly(EBAM-co-NIPAM)-P) has fine texture, whereas the hydrophobic crosslinker (poly(EGDMA-co-NIPAM)-P) creates more thick and bigger texture. These differences in morphologies of the hydrogels show that not only the character of crosslinker but also initiation system plays an important role in the formation of hydrogel structures, thus in swelling and release behaviors.

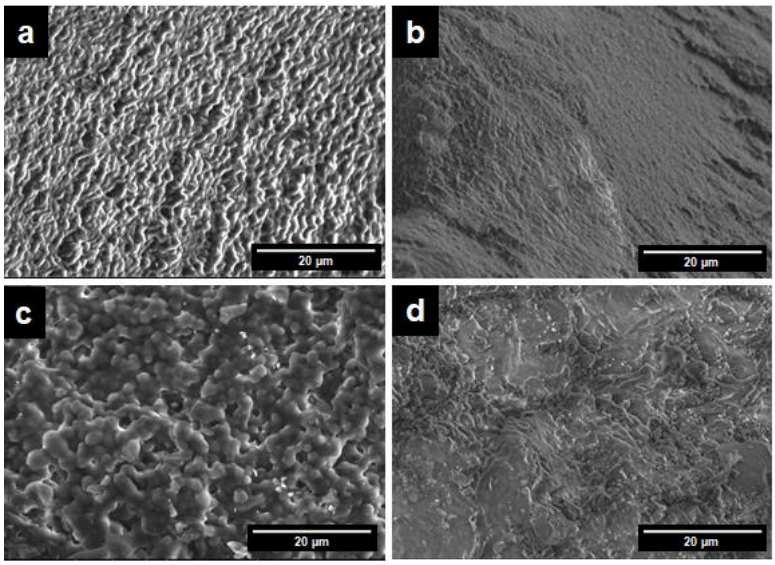

Figure 4. *SEM images of* ***(a)*** *poly(EBAM-co-NIPAM)-P;* ***(b)*** *poly(EBAM-co-NIPAM)-T;* ***(c)*** *poly(EGDMA-co-NIPAM)-P;* ***(d)*** *poly(EGDMA-co-NIPAM)-T.*

D. CYTOTOXICITY OF MONOMERS AND THERMORESPONSIVE HYDROGELS

Figure 5 shows the results of cytotoxicity experiments with the monomers and hydrogels with concentrations ranging from 1 mg/mL to 10 mg/mL for monomers and 1 mg/mL to 20 mg/mL for the hydrogels. Cells showed at least 70% viability in the presence of 1 mg/mL of each monomer solution (Figure 5a) whereas for the hydrogels with 1 mg/mL concentrations, the viabilities of the cells were at or above 100% as compared to controls (Figure 5b). However cellular viability reduced with increasing concentration of the monomers, a severe decrease in the cell viability was observed especially with 10 mg/mL concentrations. On the other hand, the viability of cells was more than 50% in poly(EBAM-co-NIPAM)-P, poly(EGDMA-co-NIPAM)-P, poly(EBAM-co-NIPAM)-T with 10 mg/mL concentrations, whereas cells showed less than 30% viability in poly(EGDMA-co-NIPAM)-T with 10 mg/mL concentration.

IC50 (50 % cell growth inhibition) values were calculated by using the curve constructed by plotting cell survival (%) versus hydrogel concentration (mg/mL). IC50 values are 10,44 mg/mL, 15,58 mg/mL and 7,14 mg/mL for poly(EBAM-co-NIPAM)-P, poly(EGDMA-co-NIPAM)-P, poly(EGDMA-co-NIPAM)-T, respectively. For poly(EBAM-co-NIPAM)-T, IC50 was not calculated as cells show more than 60% cell viability even with a concentration of 20 mg/mL of the hydrogel whereas the monomer of this hydrogel (EBAM) shows less than 10% viability at a concentration of 5 mg/mL.

In vitro cytotoxicity results revealed that poly(EBAM-co-NIPAM)-T which was synthesized by thermal polymerization is the most biocompatible hydrogel confirming that the initiation system may change biocompatibility properties of the hydrogels even the same monomer and crosslinker were used. Cells treated with 20mg/ml of poly(EBAM-co-NIPAM)-P which was synthesized by photopolymerization show almost 30% cell viability while the cells treated even with 40 mg/mL concentration of poly(EBAM-co-NIPAM)-T still show more than 60% viability (data not shown). The effect of initiation system on cell viability is also evidenced by the cell viability differences of

poly(EGDMA-co-NIPAM)-P and poly(EGDMA-co-NIPAM)-T as the hydrogel which was synthesized thermally becomes more toxic with increasing concentrations.

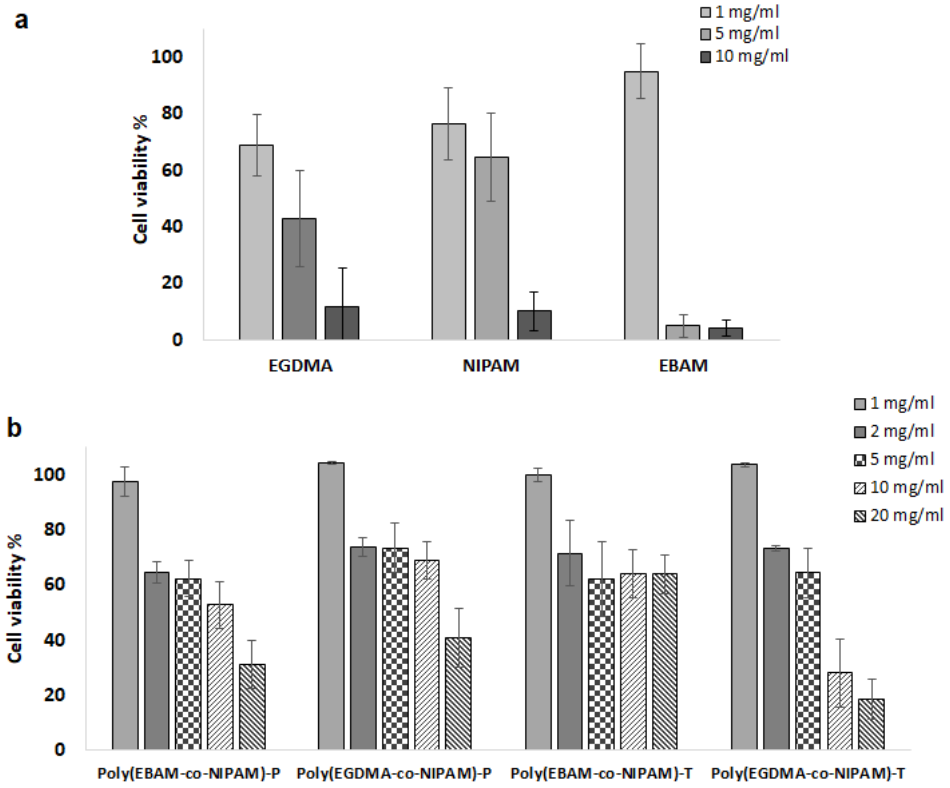

Figure 5. *Cell viability values of the monomers* ***(a)*** *and thermoresponsive hydrogels* ***(b)***.

IV. CONCLUSION

The aim of the study was to compare the effects of the initiation system and the crosslinker type on the release, swelling behavior, morphology and the biocompatibility properties of the hydrogels. Different thermoresponsive hydrogels were produced by using EGDMA and EBAM as crosslinkers via photo and thermal initiation systems. The hydrogels were characterized by FTIR spectroscopy and SEM. The hydrogels synthesized with EBAM demonstrated higher swelling ratio than those synthesized with EGDMA, however there was not a significant difference between release profiles of hydrogels. Cytotoxicity experiments revealed that the initiation system and the crosslinker type may affect the biocompatible properties of the polymers. Poly(EBAM-co-NIPAM)-T, synthesized by thermal polymerization, showed the best biocompatible character indicating that it may be further studied to use in biomedical applications such as tissue engineering and drug delivery systems.

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