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Fabrication of alginate-based stimuli-responsive, non-cytotoxic, terpolymric semi-IPN hydrogel as a carrier for controlled release of bovine albumin serum and 5-amino salicylic acid

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ABSTRACT

Herein, we report a functionalized alginate(Alg)-based terpolymeric semi-interpenetrating (semi-IPN) hydrogel, synthesized via free radical polymerization for the delivery of bovine albumin serum (BSA) and 5-amino salicylic acid (5-ASA). To improve mechanical properties, and to modulate surface morphology of Alg, 2-hydroxyethyl acrylate (HEA) was grafted on alginate and then crosslinked using poly(ethylene glycol) diacrylate (PEGDA). The probable structure and compositions of the synthesized semi-IPN terpolymer were identified by FTIR, ^{1}H-HR-MAS NMR, and TGA analyses. Achievement of equilibrium swelling state (ESS) and higher elastic modulus values confirmed terpolymer gel formation in aqueous media. Differences in the ESS of the prepared gel at pH 2.5 and 7.4 signify its stimuli-responsive behaviour. The influence of PEGDA on swelling, mechanical properties, surface morphology, cell viability and proliferation, and BSA and 5-ASA delivery were characterized. SEM images show that higher % PEGDA resulted in smaller sized pores in the gel network. Texture analyses demonstrate that hardness, adhesiveness and chewiness of the gel were enhanced at higher PEGDA concentrations. Increases in PEGDA concentration also induced increases in osteoblastic cell viability and higher rates of cell proliferation compared with gels containing lower concentrations of PEGDA. The release results indicate that the gels containing higher concentrations of PEGDA more sustainably release BSA and 5-ASA at 5 days and 30 h, respectively. The experimental data revealed that the synthesized terpolymeric semi-IPN hydrogel may have useful biomedical applications, especially as a carrier of protein (BSA), or 5-ASA (a therapeutic option for conditions of the colon such as Crohn's Disease and Ulcerative Colitis).

1. Introduction

In the past few years, polymeric hydrogels have achieved noteworthy attention in several biomedical applications [1–5] from tissue engineering (e.g., bone regeneration and wound healing) [1,6], to drug delivery [7–9]. Much of this interest in polymeric hydrogels is driven by their: i) high water content potential, ii) tuneable chemical and physical properties, and iii) capacity for encapsulation of peptides/proteins, cells, and therapeutic agents [10]. Polymeric hydrogels are semi-solid, hydrophilic, physically or chemically crosslinked 3-D networks of natural and/or synthetic polymers that retain large volumes of water [1,2,11]. Compared to synthetic polymer-based hydrogels, natural polymer-based hydrogels have unique potential for biomedical applications because of their biocompatibility, biodegradability, non-immunogenic properties, injectability, and abundance in nature [2,12]. Additionally, the presence of many functional groups (e.g., –OH, NH_2, –COOH) within their chemical structures facilitates chemical modifications with bioactive molecules or drugs to modulate physical/chemical/biological properties suitable for biomedical applications [12]. The potential suitability of a pharmaceutical agent and utility for treating a disease or condition depends not only on the therapeutic efficacy of the treatment, but also the mode of administration and the nature of the therapeutic carrier [7]. A proficient carrier can reduce adverse side effects, poor patient compliance associated with uncontrolled release and can improve the bioavailability of drugs/proteins by controlling the release rate of drugs/proteins [2,7]. Despite several advantages of natural polymers, especially biopolymers, their water-solubility creates a big obstacle for use as efficient matrices for tissue engineering and as controlled/sustained therapeutic carriers [2]. Fortunately, there are easy methods (e.g., grafting and crosslinking) for

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chemical modification to tune the physical and chemical properties of biopolymers [2]. In contrast, chemically cross-linked hydrogels have greater mechanical strength and gel strength than physically cross-linked hydrogels [11]. However, the use of excessive amounts of crosslinker can be potentially toxic due to the properties of crosslinkers used for biomedical applications [2,11]. Consequently, one of the biggest challenges for chemically crosslinked hydrogel synthesis is the development of novel, non-cytotoxic, and proficient biocompatible hydrogels with improved mechanical properties and modified swelling potentials [11]. On the other hand, among stimulus-responsive hydrogels, pH-sensitive hydrogels have a special importance in drug delivery owing to pH variations in the human body [7]. Sensitivity to pH means that hydrogels can be a triggered delivery matrix for targeted drug delivery [7].

Drug delivery-related applications of hydrogels have been employed using different types of natural and synthetic polymers [7,8,11,13–21]. However, to meet the needs of pharmaceutical applications (e.g., improved drug bioavailability, compatibility between carrier and drug, and biocompatible matrix), the development of new hydrogel remains relevant for sustained/controlled drug release. The aim of the study is to synthesize alginate-based stimuli-responsive terpolymeric hydrogels using free radical polymerization, mainly to remove the toxicity associated with crosslinker and monomer for biomedical applications. Alginic acid is a natural polysaccharide with repeating units of β-D-mannuronic acid and α-L-guluronic acid [22], and widely used in biomedical applications [22]. Sodium alginate is the salt of alginic acid which can make ionic gel quickly in the presence of divalent cations (e.g., calcium ions) through ionic interaction [22–25]. Alginate hydrogels have been extensively employed in tissue engineering, drug/gene delivery, cell encapsulation, wound healing, 3D bioprinting and more [22–26]. Due to a high degradation rate in cell culture and reduced mechanical properties, a single component alginate hydrogel confines its practical biomedical applications [22]. Furthermore, ionic gels are disrupted in basic medium, leading to the uncontrolled release of therapeutic agents [24,25] and limited application as controlled drug carriers. To overcome these disadvantages, we: i) chemically modified an alginate (Alg) moiety through graft polymerization of 2-hydroxyethyl acrylate (HEA) to overcome water solubility and, ii) added different amounts of poly(ethylene glycol) diacrylate (PEGDA) as a crosslinker to modulate mechanical properties and drug release profile of the terpolymeric gel system. The combine advantages of alginate and synthetic compounds (HEA as monomer and PEGDA as crosslinker) will be the ability to tune mechanical properties, microstructure, porosity, biological functions of biocompatible and biodegradable biopolymers [10], and biological activity of synthetic polymers [10]. The porosity of a hydrogel is a key parameter for biomedical applications as it: i) helps mediate interactions with cells and the surrounding tissues, ii) acts as a channel to transport bioactive cues to cells developing within the hydrogel, and iii) endorses cellular permeation and new tissue formation [10]. Furthermore, porosity also controls the swelling behaviour and the release rates of therapeutic agents from the hydrogel [2,9].

In the present study, we have synthesized a terpolymeric semi-interpenetrating (semi-IPN) of biopolymer-alginate and synthetic compounds (HEA and PEGDA) using graft polymerization and crosslinking processes by free radical polymerization technique. The effects of bifunctional species (PEGDA) on the physical properties (i.e., porosity and swelling), mechanical properties, and MC3T3 cell viability and proliferation of terpolymeric gels have been investigated. Additionally, the in vitro release behaviours of model proteins (bovine albumin serum) and a drug with applications for disorders of the colon (5-amino salicylic acid) from the semi-IPN hydrogel have been studied at pH 2.5/7.4 and 37 °C, and the impact of PEGDA on drug release rates has been examined. To the best of our knowledge, this is the first reports on biocompatible and pH-responsive terpolymeric semi-IPN hydrogel of alginate, HEA and PEGDA for the delivery of bovine albumin serum and 5-amino salicylic acid.

2. Materials and methods

2.1. Materials

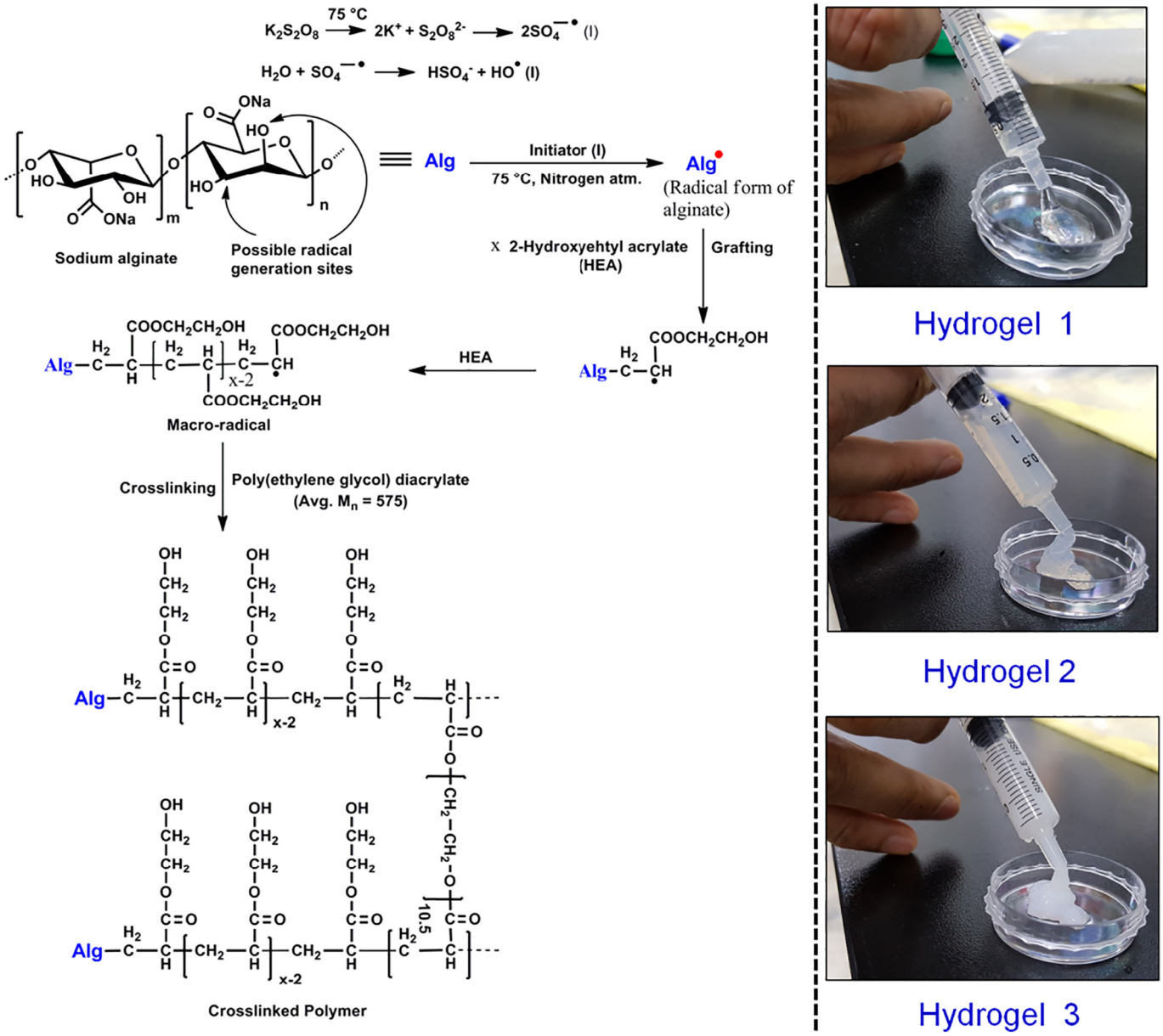
Dimethyloxalylglycine (DMOG, Cayman Chemical Company, USA), fetal bovine serum (FBS, Biotechnics Research, Mission Viejo, CA, USA), penicillin-streptomycin (Lonza, Seoul, Korea), cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan), live & dead viability/cytotoxicity kit for mammalian cells (Invitrogen, Carlsbad, CA, USA) and bromodeoxyuridine (BrdU, Roche, Germany) were procured for experiments. Other chemicals such as alginic acid sodium salt (Alg, from brown algae, medium viscosity, Mw = 818,258 Da, PDI = 5.412), potassium persulfate (KPS), 2-hydroxyethyl acrylate (HEA), poly(ethylene glycol) diacrylate (PEGDA, Avg. molecular weight ~ 575 Da), bovine serum albumin (BSA, molecular weight ~ 66 kDa), 5-Aminosalicylic acid (5-ASA, M.W. = 153.14 g/mol), alpha minimum essential medium (α-MEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), neutral red and the staining reagents for H&E and MT were bought from Sigma Aldrich (St. Luis, MO, USA, Germany and China)]. Distilled water was employed for all experiments.

2.2. Synthesis

First, a 50 mL aqueous solution was prepared by dissolving 0.25 g of alginate (Alg; 6.31×10^{-4} mol taking into consideration of the molecular weight of one unit as shown in Scheme 1) in a 2-neck round bottom flask (Duran, Germany) using digital glass oil bath (LK Lab Korea, Korea) at 50 °C and 400 rpm for 6 h. Then, the temperature was increased to 75 °C and nitrogen gas was purged into the solution for 30 min to generate inert atmosphere. Next, 5 mL aqueous solution of the initiator KPS (0.004 g, 0.147×10^{-4} mol) was added using a syringe to the alginate solution. After 20 min, 3 mL of HEA (2.84×10^{-2} mol) as monomer was mixed with the solution. Once the viscosity of the solution increased, three different volumes of a bifunctional prepolymer [e.g., 0.25 mL (4.87×10^{-4} mol), 0.50 mL (9.74×10^{-4} mol), and 1 mL (19.48×10^{-4} mol) of PEGDA] were separately added. After the addition of PEGDA, the reaction was continued for 2 h. The product was purified through dialysis in distilled water at 25 °C for 2 days. Finally, the purified samples were dried at lyophilizer at −56 °C for 7 days.

2.3. Characterizations

Molecular weight of the alginic acid sodium salt (Alg, from brown algae, medium viscosity, Sigma Aldrich) was determined relative to PEG/PEO standards on a gel permeation chromatography (Model: Tosoh EcoSEC HLC-8320 GPC) equipped with 2 × Tskgel GMPW × l + Tskgel G2500PW × l and RI detector at Korea Polymer Testing and Research Institute (KOPTRI, Seoul, Korea). For this study, 3 mL of alginate solution (3 mg/mL) at buffer pH 7 was injected with a rate of 1 mL/min. FTIR spectra of the compounds were recorded using an ATR-FTIR spectrometer at a range of 650–4000 cm^{-1} (Model: Travel IR, Smiths Detection, USA). The ^{1}H NMR spectra of sodium alginate were carried out with nuclear magnetic resonance (NMR) spectrometer (Model: DD2 700, Agilent Technologies-Korea, USA). The HR-MAS NMR (proton) analyses of three hydrogels were executed in Korea Basic Science Institute (Seoul, Korea) using AVIII 700 MHz NMR spectrometer (Bruker Instruments, Inc., Germany). A 4 mm-TXI HR-MAS (high-resolution magic angle spinning) probe head was used and the spinning rate was 6 kHz. Gel samples were prepared by adding D_2O in dried terpolymer and 50 μL gel was inserted with a 4 mm ZrO_2 rotor. The thermogravimetric analyses were accomplished using a thermogravimetric analyser (TGA, Model: DTG-60, Shimadzu, Japan) at nitrogen atmosphere, and a scan rate of 5 °C/min. The surface and cross-section morphology of three dried hydrogels were examined using scanning electron microscopy (SEM; Model: TESCAN VEGA3, Tescan Korea). The



Scheme 1. Probable mechanism and digital images of alginate, HEA and PEGDA based terpolymeric semi-IPN hydrogel.

release of DMOG and BSA were measured using a UV–Vis spectrophotometer (Model: BioMATE 3, Thermo Scientific, USA). Image *J* software has been used to measure the sizes of the pores in the hydrogels, where thirteen pores has been selected to get average sizes of the pores. The data has been represented as Avg. ± S.D.

2.4. Texture analysis

Mechanical properties (Hardness, cohesiveness, adhesiveness, springiness, resilience, and chewiness) of the three grades terpolymeric semi-IPN hydrogel were measured using Stable Micro Systems (Model: TA.XT plus texture analyser, Surrey, UK). Rectangular shaped terpolymer gels with a dimension of 1 × 1 × 0.5 cm^3 was used for the experiment, where maximum strain was 30%. A 50 N load cell was fitted for this experiment. The pre-, test-, and post velocities were all 1 mm/s, and the probe was located on the surface of the gel. To examine the changes of the mechanical properties of the terpolymeric semi-IPN hydrogels after autoclave sterilization, mechanical properties were also studied after autoclave of the gel samples. The experiment was done two times and data were represented as Average ± S.D.

2.5. Swelling study

Swelling studies of three dried crosslinked polymers were carried out gravimetrically. In brief, 1 mL of dialyzed crosslinked polymers were kept in a 24 well-plate and dried in a lyophilizer for 96 h. Next, the dried crosslinked polymers were immersed in 100 mL of buffer solutions (pH 2.5 and 7.4) at 37 °C for 15 h. After a regular interval, the wet terpolymers were removed from buffer solutions and surface water was wiped with tissue paper. Next, the wet crosslinked polymers were re-weighed until an equilibrium of their weights was reached. The % swelling was calculated by the Eq. (1):

$$Swelling\ (\%) = \frac{W_{wtp} - W_{dtp}}{W_{dtp}} \times 100(\%) \quad (1)$$

where, W_{wtp} and W_{dtp} is the weight of wet crosslinked polymer and dried crosslinked polymer, respectively. To detect the pH-responsive nature of the crosslinked polymer, swelling study was done in acidic

(pH 2.5) and basic media (pH 7.4). Besides, pH 2.5 and physiological pH i.e. pH 7.4 were selected to observe the behaviour of the hydrogel in these pHs for its possible application in drug delivery and tissue engineering.

2.6. In vitro cell study

2.6.1. In vitro cytotoxicity study of terpolymeric semi-IPN hydrogel

The in vitro toxicity of the three grades of terpolymeric semi-IPN hydrogel were assessed based on their impact on cell structures like mitochondria, lysosome and DNA by thiazolyl blue tetrazolium bromide (MTT), Neutral Red and bromodeoxyuridine (BrdU) assays, respectively [24,25,27]. For this study, extract solutions of autoclave-sterilized teflon sheet (diameter = 1 cm), latex (diameter = 1 cm), and three terpolymeric gel films (diameter = 1 cm) were incubated in 1.5 mL culture media for 3 days. Teflon and latex were used as positive, and negative controls, respectively. Alternatively, MC3T3 cells were cultured in a 96-well plate with a density of 1×10^4 cells/well for 24 h. After 24 h, medium was discarded and 100 μL of extract solutions were added into each well and incubated for another 24 h. The cytotoxicity analyses were performed by MTT, BrdU and neutral red assays according to our previous reports [24,25,27].

2.6.2. Osteoblast cell proliferation study on the surface of the terpolymeric semi-IPN hydrogel

In vitro osteoblast cell (MC3T3) proliferation studies on the surfaces of three grades of terpolymeric semi-IPN hydrogel films were conducted to evaluate gel biocompatibility. First, 0.1 mL of dialyzed three grade gels were put on coverslips and then stored in an oven for 72 h. Next, the round shape films (d = 1 cm) containing coverslips were sterilized by autoclave at 121 °C for 15 min. 1×10^5 osteoblast cells derived from *Mus musculus* mouse (MC3T3, Sigma Aldrich) were seeded on the surfaces of gel films in a 24 well plate, and then incubated for 20 min at 5% CO_2 and 37 °C. Subsequently, 1 mL of α-MEM media containing 10% fetal bovine serum and 1% penicillin-streptomycin was put on each well and incubated for 7 days. After every 24 h, culture medium was changed. After 1, 3 and 7 days, the degrees of cell proliferation were calculated using a CCK-8 assay according to the manufacturer's protocols [24,25,27]. The live/dead assay was performed using ethidium homodimer-1/calcein AM staining according to our previous protocol [24,25,27]. The images of MC3T3 cells on gel films were captured by a fluorescence microscope (Leica DMLB, Wetzlar, Germany).

2.7. Incorporation of protein (bovine albumin serum) and drug (5-amino salicylic acid) into the semi-IPN hydrogel and in vitro release studies

A total of 2 μmol of bovine albumin serum (BSA) and 5-amino salicylic acid (5-ASA) were separately mixed with 2 mL of three grades of semi-IPN hydrogel in a 24 well plate for 30–45 min by a spatula to make homogeneous blends. Then, the BSA/5-ASA incorporated terpolymeric gel samples were dried in a lyophilizer at −56 °C for 72 h. The in vitro BSA and 5-ASA release studies from dried loaded gels were executed at pH 2.5/7.4, and 37 °C. For this study, the BSA/5-ASA loaded gels were put in teflon flasks containing 100 mL of buffer media (pH 2.5 and 7.4) at 37 °C. After different time intervals, aliquots were removed from flasks and absorbances were measured by UV–Vis spectrophotometer (Model: BioMATE 3, Thermo Scientific, Madison, USA). The % BSA and 5-ASA release were determined compared with standard solutions. To detect the pH-responsive drug release nature and effect of swelling on drug release from the terpolymer pH 2.5 and 7.4 were chosen. Besides, pH 2.5 and pH 7.4 were used as simulated gastric fluid, and intestinal fluid/physiological pH to observe release behaviour of colon targeted drug (5-ASA) release and model protein (BSA) for the applicability of the terpolymeric semi-IPN hydrogel towards drug delivery and tissue engineering.

2.8. Statistical analysis

All data are presented as mean ± standard deviation. Statistical analysis was conducted with one-way and multi-way ANOVA by using the SPSS 18.0 program (ver. 18.0, SPSS Inc., Chicago, IL, USA). Comparisons between two groups were performed by *t*-test and significant differences were considered at $p < 0.05$.

3. Results and discussion

3.1. Synthesis of alginate-based semi-IPN hydrogel

A semi-IPN hydrogel composed of alginate (Alg), 2-hydroxyethyl acrylate (HEA), and poly(ethylene glycol) diacrylate (PEGDA) was synthesized via free radical polymerization using potassium persulfate (KPS) as initiator. Scheme 1 is the plausible mechanism of the synthesis. It is presumed that, in the presence of heat, KPS dissociates into sulphate ion radicals, which further reacts with solvent (H_2O) and form hydroxyl radicals [28,29]. These radicals can abstract hydrogen atoms from (i) carbon main skeleton of alginate backbone [30], and (ii) –OH groups present in the alginate backbone [30–35] and form alginate macro-radicals. These radicals of alginates react with the HEA and initiates grafting of HEA towards alginate backbone [30–35]. Grafting of HEA followed by polymerization offers improved entanglement to the polymeric network [30]. In addition, it also obstructs leaching of alginate moiety during swelling of the polymer [30].

When PEGDA was added, it is assumed that due to the presence of two unsaturated groups, PEGDA can crosslink Alg/HEA macro-radicals and formed semi-interpenetrating polymer network (Scheme 1). A semi-interpenetrating polymer network (Semi-IPN) is a biphasic polymeric system where one phase is cross-linked in presence of the other phase [30]. Here, three grades of semi-IPNs are named as hydrogel 1, hydrogel 2 and hydrogel 3, on the basis of three different amounts of PEGDA.

3.2. Characterizations

Fig. 1 describes the GPC study result of sodium alginate (Alg) used for the synthesis of terpolymeric hydrogel. It is observed that the time for peak top was 19.383 min (Fig. 1a). The number average (Mn) and weight average (Mw) molecular weights were 151,187 and 818,258 Da, while PDI value was 5.412 (Fig. 1b).

In the FTIR analysis, alginate showed peaks at 3254, 1597, 1405, 1082 and 1026 cm^{-1} because of the −O−H, C═O, C−O, asymmetric and symmetric C-O-C stretching frequencies, respectively (Fig. 2a).

The peaks at 3428, 2953, 2885, 1715, 1633, 1274,1188, and 1057 cm^{-1} in the FTIR spectrum of the HEA are because of the stretching frequencies of O−H, C−H, C═O, C═C bonds, C−O, and C-O-H stretching frequencies, respectively (Fig. 2b). In the FTIR spectrum, PEGDA demonstrates peaks at 2870, 1723, 1633, 1271, 1192, and 1100 cm^{-1} are owing to the C−H, C═O, C═C, C−O, asymmetric and symmetric C-O-C stretching frequencies, respectively (Fig. 2c). Whereas, in the FTIR spectra of three dried semi-IPN gels (Fig. 2d–f), the absence of peak at 1633 cm^{-1} indicates conversion of sp^2 carbons to sp^3 carbons of both HEA and PEGDA, which support the reactivity of HEA/PEGDA as well as the grafting of HEA and crosslinking through PEGDA followed by polymerization. The peaks between 2945 and 2881 cm^{-1} are due to C−H stretching frequency, while the peaks between 1723 and 1724 cm^{-1} and 1232–1243 cm^{-1} are because of the stretching frequencies of C═O and C−O bonds of ester groups, signifying the presence of the HEA and PEGDA units in the gel network (Fig. 2d–f). Peaks at 1614–1615 cm^{-1} are due to the C═O of the carboxylate group of alginate, whereas, peaks between 1071 and 1161 cm^{-1} are owing to the asymmetric and symmetric C-O-C stretching frequencies (Fig. 2d–f).

In the ^1H NMR analysis, alginate (Fig. 3a) showed chemical shifts at

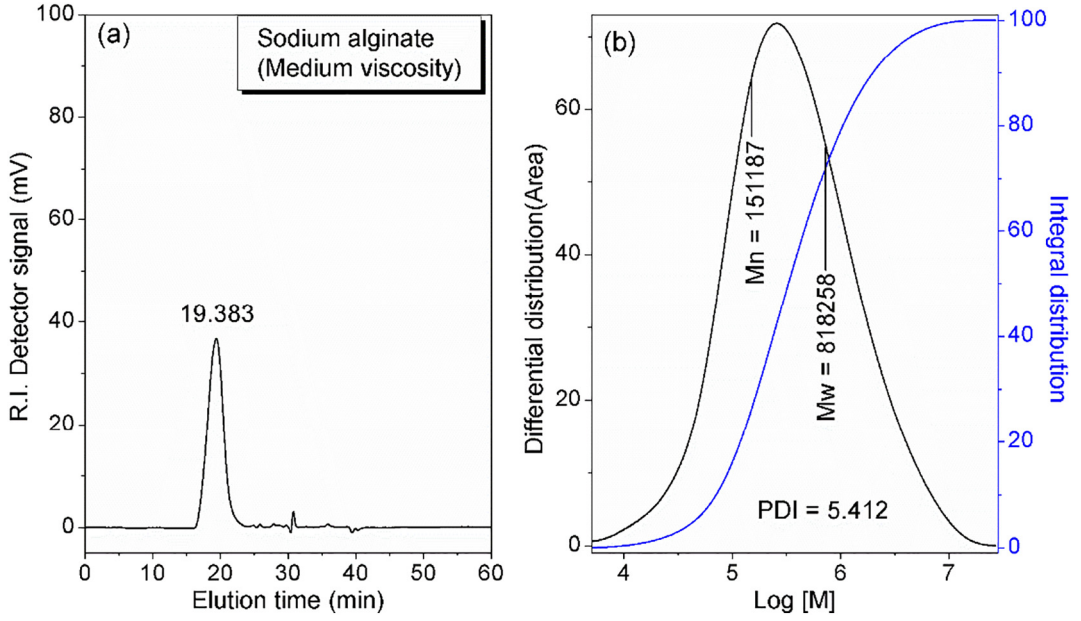


Fig. 1. GPC study results of sodium alginate (Alg).

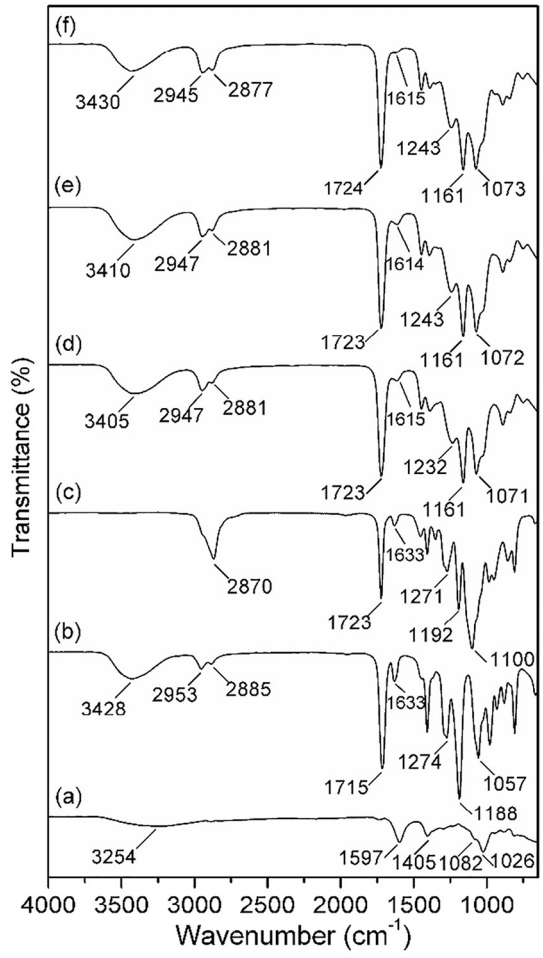


Fig. 2. FTIR spectra of (a) alginate, (b) HEA, (c) PEGDA, and dried (d) hydrogel 1, (e) hydrogel 2, and (d) hydrogel 3.

δ = 4.90, 4.51, 4.34, 3.61–4.05 ppm, which are responsible for the anomeric proton of guluronate (G1), anomeric proton of mannuronate (M1) and other ring protons (G2-G4, M2-M4), respectively [36]. In the ^{1}H-HR MAS NMR spectra of three semi-IPN gels (Fig. 3b–d), the chemical shifts between δ = 3.59, 4.01, and 4.22 ppm indicate presence of alginate in the hydrogel. The chemical shifts at δ = 3.64, 3.74, and 4.12–4.15 ppm are because of the characteristic protons of the HEA and PEGDA units (i.e. H14, H9, and H8/H13 protons), respectively. The absence of chemical shifts between 5.8 and 6.4 ppm indicates the reactivity of unsaturated carbons protons of both HEA and PEGDA in the reaction condition [37,38]. Meanwhile, the new chemical shifts appeared between δ = 1.59, 1.75, and 3.84 ppm are due to the H6 and H15 protons which confirmed the grafting of HEA in both main carbon backbone and –OH groups of alginate. Chemical shifts at δ = 1.96, 2.39, 2.46 ppm for H10, H7/11 and H12 imply that the polymerization of HEA and crosslinking through PEGDA occurred successfully in the gel network. Although it is complicated to determine the % grafting and % crosslinking using ^{1}H NMR spectra of alginate and semi-IPN gel due to the appearance of the peaks of multiple compounds (HEA and PEGDA) in very proximity. However, considering the integration of chemical shift value at δ = 3.59 as base value (I_{Alg} = 1.00) for one proton of alginate, the integration values of H6 and H10 protons (of HEA) were used to calculate % total grafting. While, the integration value of H14 protons (of PEGDA) was used to calculate % crosslinking.

The % grafting [$0.5*I_{H6}/I_{Alg} + 0.5*I_{H15}/I_{Alg}$) × 100%] are 2083%, 2081%, and 2035% for hydrogel 1, 2, 3, respectively. Similarly, the % crosslinking [(I_{H14}/I_{H9}) × 100%] are 18.1%, 48.7%, and 93.8% in hydrogel 1, 2, 3, respectively. The progressive increases of integration values for H14 protons of PEGDA confirmed the presence of three different amounts of crosslinker (PEGDA) in three grades of hydrogel (Fig. 3b–d).

Fig. 4 represents the TGA results of three grades of dried hydrogel. In the plots, the first weight loss (180 °C–344 °C) signifies the breakdown of alginate network [25]. The second weight loss zone, on the other hand, is due to the breakdown of crosslinked polymer network. As shown in Fig. 4, it was observed that higher % PEGDA-containing terpolymer (hydrogel 3) showed lower % weight loss, and lower % PEGDA containing terpolymer (hydrogel 1) exhibited higher % weight loss. The results indicate that more PEGDA in the hydrogel network enhanced the thermal stability of the synthesized polymer. This can be explained by the fact that more PEGDA increased the association of PEGDA molecules in the polymeric network through covalent bonding, which makes the network stronger when compared with polymers containing lower concentrations of PEGDA. We have quantitatively measured the

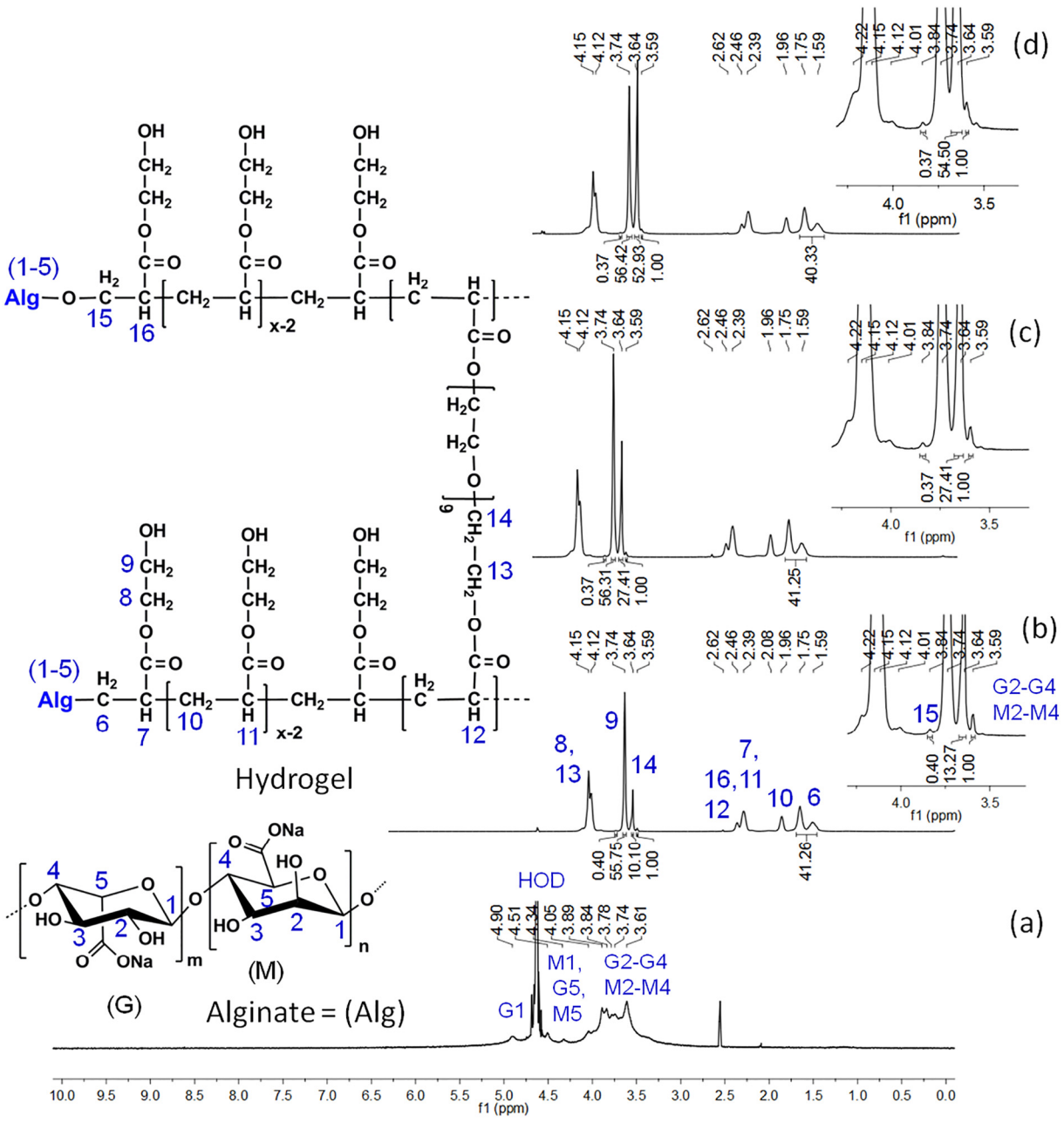


Fig. 3. (a) ^{1}H NMR spectrum of alginate, and (b-d) ^{1}H HR MAS NMR spectra of semi-IPN hydrogel 1, 2, 3, respectively (D_2O was solvent).

temperatures of 20%, 50% and 80% weight loss of the three grades of hydrogel. It is noticed that the temperatures of 20%, 50% and 80% weight loss of hydrogel 1 were 301.8, 399.1, and 437.1 °C, respectively. The temperatures of 20%, 50% and 80% weight loss of hydrogel 2 were 315.8, 397.0, 426.3 °C, respectively. The temperatures of 20%, 50% and 80% weight loss of hydrogel 3 were 315.2, 389.9, 426.1 °C, respectively.

The surface morphologies of three grades of dried semi-IPN hydrogel are shown in Fig. 5. Each image confirms that polymeric gels have porous morphology and the sizes of pores depend on the concentration of PEGDA in the gel network. Pore sizes remain within micrometre ranges. It is clear from the SEM images of the gels that higher % PEGDA induced smaller sized pores. This can be explained by the fact that as the amount of PEGDA increased, more PEGDA molecules polymerize resulting in more crowded gel networks with small sized pores. The average sizes of the pores of hydrogel 1, hydrogel 2, and hydrogel 3 were 9.4 ± 3.1 μm, 5.2 ± 1.1 μm, and 1.8 ± 0.4 μm, respectively.

3.3. Texture analysis

The mechanical properties (e.g., hardness, cohesiveness, adhesiveness, springiness, resilience, and chewiness) of the three grades of terpolymeric semi-IPN hydrogel were analysed using a texture analyser. These analyses were undertaken to observe the effect of PEGDA concentration, and autoclave on the mechanical properties of the prepared gels. Hardness is associated with the strength of the gel structure under compression, chewiness refers to the energy requisite to crunch a material to a state ready for swallowing [39]. From Fig. 6, it is observed that both hardness (Fig. 6a) and chewiness (Fig. 6f) are significantly affected by the amount of PEGDA and autoclave. As the concentration of PEGDA in the gel was increased, PEGDA linked the graft networks and itself self-associates through polymerization resulting in increased intramolecular interactions within the hydrogel network, enhanced hardness and chewiness.

However, hardness and chewiness decreased after autoclave.

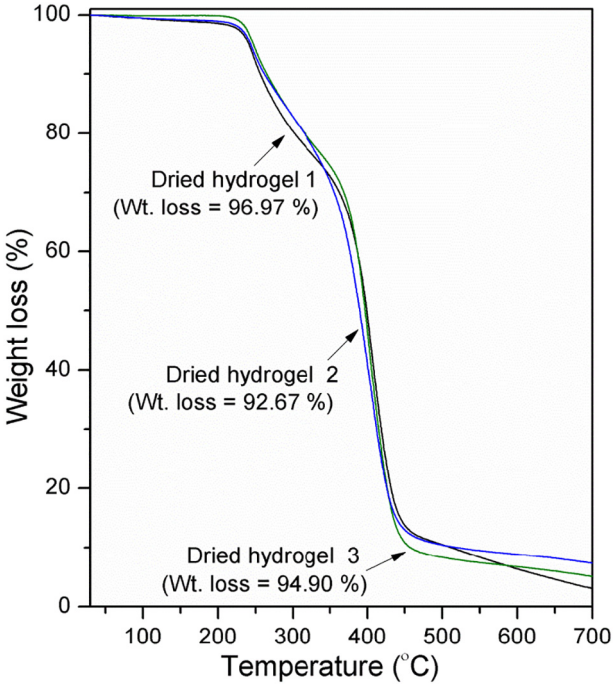


Fig. 4. Thermogravimetric analyses results of three dried terpolymeric semi-IPN hydrogels.

Adhesiveness measures a material's capacity to adhere to the disc [40]. The results of texture analysis showed that increases in the concentration of PEGDA leads to increased adhesiveness to the hydrogel (Fig. 6b); the adhesiveness of the hydrogel was decreased after autoclave. A gel is cohesive when it adheres to itself under compressive conditions or stress. Thus, cohesiveness relates to the degree of difficulty in breaking down the gel's interior assembly [39]. Springiness, sometimes referred to as “elasticity”, is a measure of gel structure break down by initial compression [39,41]. Our results show little differences in cohesiveness of the hydrogels; similarly, springiness also slightly decreased after autoclave. Resilience is a measure of a material's capacity to deform elastically without loss of energy [42]. It is apparent that increases in the concentration of PEGDA faintly affects resilience of the hydrogel. Under autoclave, the mechanical properties decreased, which may owe to the rapture of the intramolecular interaction/association (via physical interaction) in the hydrogel network in the high temperature of autoclave. The presence of adequate hardness and adhesiveness signify the biological applicability of the terpolymeric hydrogel to tissue engineering and wounds where the gel can resist the physiological stress caused by the movement of skin (body), and in drug delivery [43].

3.4. Swelling study

The results of swelling studies of three grades of crosslinked polymer at pH 2.5/7.4 and 37 °C are presented in Fig. 7. It is obvious from the results that the terpolymers attained its equilibrium swelling state at ~14 h, which confirms the gel nature of terpolymers at pH 2.5/7.4 and 37 °C. We also note that the % swelling depends on the pH of the media, where % swelling of all grades are higher at pH 7.4 than at pH 2.5 (Fig. 7). At basic medium (i.e., pH 7.4), the acid groups of alginate unit of the gel network remain carboxylate ions, which make the matrix more hydrophilic allowing for increased absorption of water molecules. Besides, at this medium, the repulsive force between the carboxylate ions stretch the terpolymeric network. As a result, more water molecules can easily diffuse into the network, resulting in higher swelling percentages. While, at pH 2.5, carboxylate ions of alginate units of the gel protonated and form carboxylic acid groups.

This protonation causes intramolecular H-bonding, which makes the polymer network more rigid than that at pH 7.4. Thus, void space in the terpolymer decreased and absorb less water, resulting in lower swelling percentages than that at pH 7.4. The results clearly indicate the pH

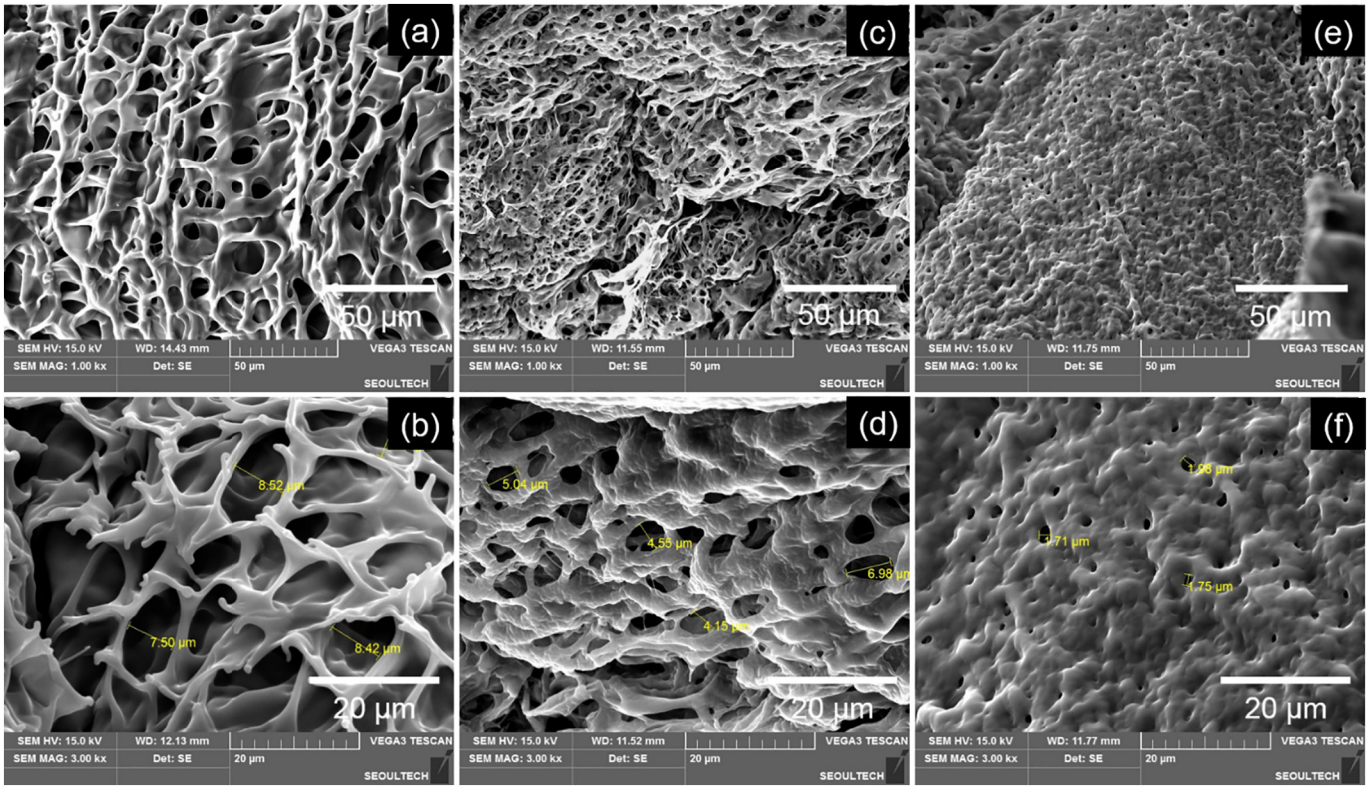


Fig. 5. SEM analyses results of dried hydrogel 1 (a, b), hydrogel 2 (c, d) and hydrogel 3 (e, f) with 1 Kx mag. and 3 Kx mag.

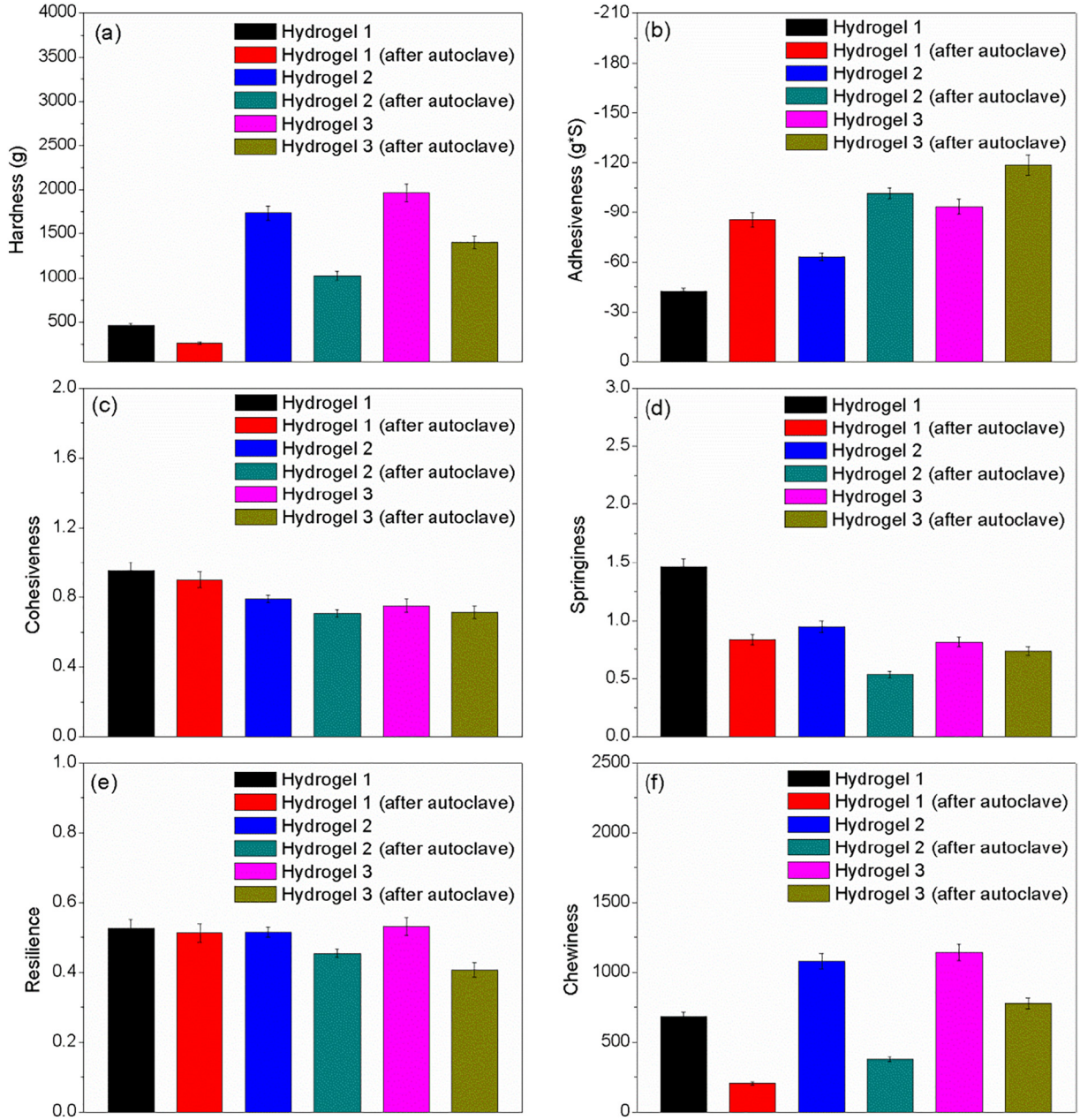


Fig. 6. Mechanical properties of three semi-IPN hydrogels before and after autoclave, obtained from texture analysis (results are displayed as Avg. ± SD, n = 2).

responsiveness of the synthesized terpolymeric semi-IPN hydrogel. Again, among all grades, the grade containing highest concentration of PEGDA (hydrogel 3), demonstrated lower swelling percentages than the other two grades (hydrogel 1 and hydrogel 2) at both media. This may be because of fewer pores and stiffer structure, formed due to the formation of intramolecular H-bonding of hydrogel 3 than those of hydrogel 1 and hydrogel 2 (Fig. 5).

3.5. In vitro cell study

3.5.1. In vitro cytotoxicity study

The results of in vitro toxicity tests [i.e., MTT, neutral red (NR) and BrdU assays against osteoblastic cells (MC3T3)] are presented in Fig. 8. The results of the MTT and BrdU assays (Fig. 8A), show that MC3T3 cell viability is higher in the presence of terpolymeric semi-IPN hydrogels films extracts than those of both positive control-teflon, and negative control-latex (MTT: 101.1 ± 4.7%, 102.2 ± 8.3%, 108.5 ± 12.2%; BrdU: 100.2 ± 3.1%, 104.4 ± 5.5%, 105.9 ± 6.9%). The NR assay results indicate that cell viability is significantly higher than that of negative control-latex (NR: 91.1 ± 9.8%, 91.7 ± 8.4%, 96.2 ± 7.5%); viability is > 90%. The florescent live/dead MC3T3 cells images shown in Fig. 8 suggest that after 24 h of different extracts addition, all cells are live, and the cell density in the teflon, and three gel extracts plates were increased (Fig. 8b, d, e, f) compared with pretreated samples (Fig. 8a), whereas, most of cells were dead in case of latex extracts. These results confirm that the terpolymeric gels are not cytotoxic. It is also obvious that as the concentration of PEGDA in the hydrogel increases, cell viability increases (Fig. 8A).

3.5.2. In vitro MC3T3 cells proliferation study

MC3T3 cells proliferation studies were performed on the terpolymeric semi-IPN hydrogel films to ascertain gel biocompatibility. Tissue culture plates were used as controls for this study. As shown in Fig. 9A, it is apparent that the optical density values increased in both control

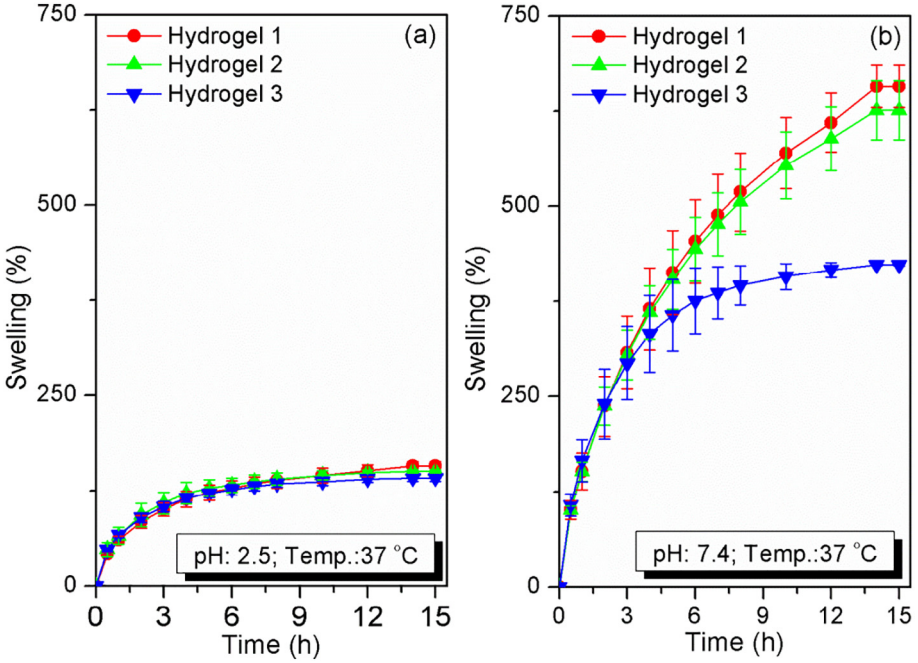


Fig. 7. Plots of swelling (%) vs. time (h) of three dried crosslinked polymers at pH 2.5/7.4 and 37 °C (results are represented as Avg. ± SD, n = 3).

and three types of terpolymeric gels over time (from day 1 to day 3 and 7).

Cells were significantly increased on all terpolymeric hydrogels films (Fig. 9A). Compared to day3/day1, rate of cell proliferation was higher for day7/day1 which indicates that hydrogels provide appropriate conditions for cell growth and proliferation. These results are also supported by imagining analyses of live/dead MC3T3 cells. The images demonstrate that cells are attached on the surface of culture plates (Fig. 9a, e, i), and on all three grades of gels films, i.e. hydrogel 1 (Fig. 9b, f, j), hydrogel 2 (Fig. 9c, g, k), and hydrogel 3 (Fig. 9d, h, l). Also seen in Fig. 9 is that optical density and rate of cell proliferation progressively increased in the gel matrix with the concentration of PEGDA. From texture analyses, it has been noted that adhesiveness increased as the concentration of PEGDA in the gel matrix increases. Additionally, it is assumed that due to the smaller pores sizes in hydrogel 3, most of the cells were grown on the surface, while there was a

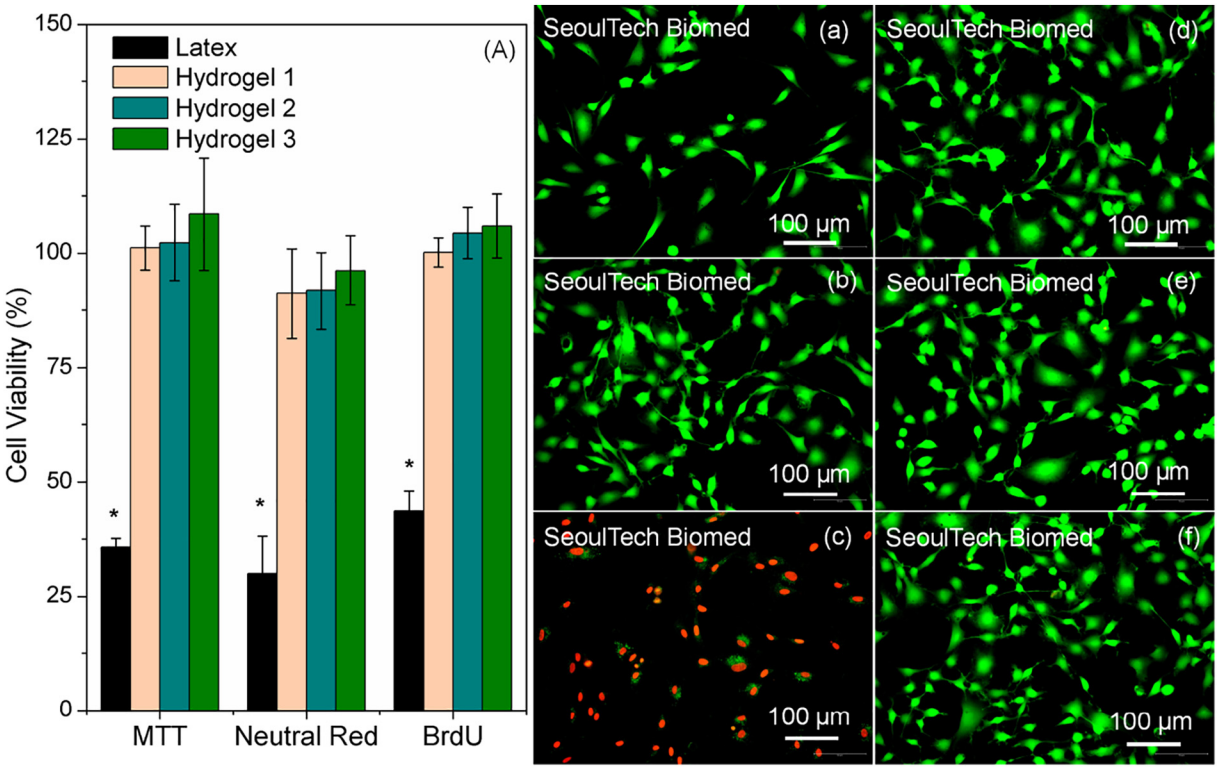


Fig. 8. (A) MC3T3 cell proliferations in presence of extracts of teflon, latex, three terpolymeric semi-IPN hydrogels, and (RHS) live/dead MC3T3 cells images: (a) before extract addition, and after addition of extracts from (b) teflon, (c) latex, and terpolymeric gel films after 1 day in vitro cell culture.

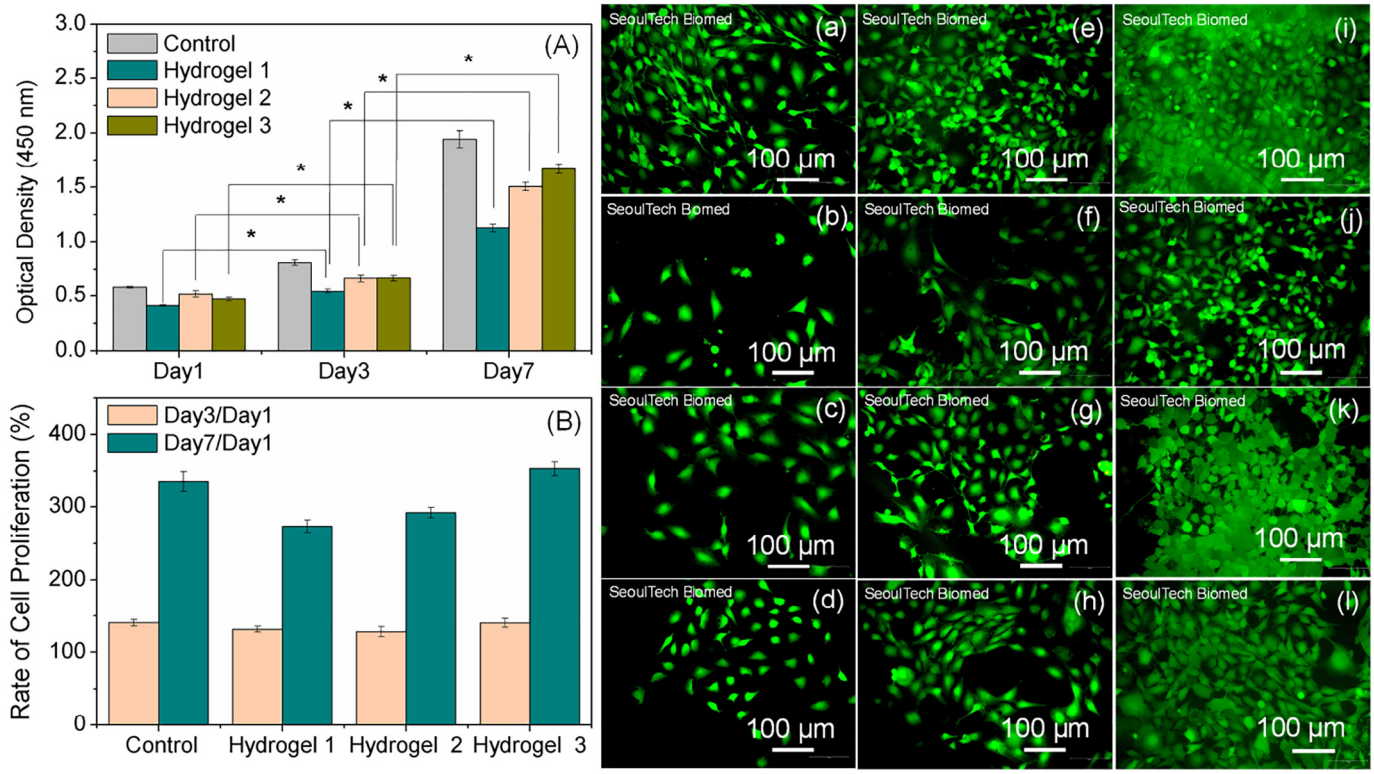


Fig. 9. (A, B) MC3T3 cell proliferation graphs, and fluorescence images of live and dead cells on the surface of polystyrene tissue culture plate (control), three terpolymeric semi-IPN hydrogel films after (a-d) day 1, (e-h) day 3, and (i-l) day 7 of in vitro culture.

decreased chance in the migration of adhered cells into the polymer network than those of the other two gels with larger pore sizes. These characteristics of hydrogel 3 assisted more cell adhesion, and provided compatible polymer network for cell growth, showed higher optical density and rate of cell proliferation. The results suggest that terpolymeric semi-IPN hydrogel are biocompatible.

3.6. In vitro protein (bovine albumin serum) and drug (5-amino salicylic acid) release studies

The results of in vitro release studies of 5-ASA and BSA from 5-ASA/BSA incorporated into three grades of semi-IPN hydrogels at pH 2.5/7.4 and 37 °C are presented in Fig. 10. Release profiles indicate the pH-dependent nature of 5-ASA and BSA release from hydrogels (Fig. 10). As described in the swelling section, the swelling percentage of terpolymers are higher in basic media (pH: 7.4) because of the formation of negative charged carboxylate ions and polymeric network stretching owing to repulsive force between those groups. Owing to the higher swelling percentage at pH 7.4, rates of 5-ASA and BSA releases were faster in this medium than at pH 7.0 (Fig. 10). On the other hand, owing to the conversion of carboxylic acid groups from carboxylate ions, and formation of rigid structure, rate of water diffusion is slower, and thus rate of 5-ASA and BSA are slower. Among three gels, it is obvious that as the concentration of PEGDA in the gel increases, rates of 5-ASA and BSA release were reduced.

It is noted that increased concentrations of PEGDA resulted in increases in gel hardness and pore size of the gel network as described above. These combined effects are responsible for the decreased rates of 5-ASA and BSA release in gels containing higher PEGDA concentrations (Fig. 10). As shown in Fig. 10, the releases rate of 5-ASA is faster than BSA in all gel grades in both media. It is expected that the physical interaction (e.g., H-bonding) between hydrogel and low molecular weight (153.14 g/mol), smaller sized 5-ASA drug (Fig. 10A and B) will be stronger than that with high molecular weight protein-BSA (~66,000 g/mol), which may be one factor impacting drug/protein release. However, owing to shorter mean dissolution time, a low molecular weight drug showed faster release than that of a high molecular weight drug [44,45]. Because of these combined effects, 5-ASA diffused through the gel layer more readily than that of BSA. The release results demonstrate that the synthesized terpolymeric semi-IPN hydrogel can release colon-specific drug (5-ASA) and protein (BSA) throughout > 30 h, and 5 days, respectively (Fig. 10). This results also imply that the semi-IPN hydrogel could serve as a controlled release matrix for both 5-ASA and BSA.

4. Conclusions

A hybrid semi-IPN hydrogel of biopolymer (alginate) and synthetic monomers (HEA and PEGDA) was successfully synthesized via free radical polymerization. To improve mechanical properties, and to modulate surface morphology of water soluble sodium alginate, HEA was grafted on alginate, and then crosslinked via bi-functional PEGDA followed by polymerization, which resulted formation of the terpolymeric semi-IPN. The probable structure and composition of the synthesized polymer were ascertained by FTIR, ^{1}H-HR-MAS NMR, and TGA analyses. The achieved equilibrium swelling state and offered higher elastic modulus value in rheology study established the gel making capability of the synthesized terpolymer in aqueous medium. Different swelling ratio percentages of the prepared gel at pH 2.5 and 7.4 signified its stimuli responsive characteristics. It was observed that increases in the concentration of PEGDA in the gel network reduced the sizes of pores. Increases in the concentration of PEGDA significantly enhanced the mechanical properties (e.g., hardness, adhesiveness and chewiness) of the hydrogel. Because of the presence of small sized pores and better mechanical properties, gels with increased concentrations of PEGDA resulted in increased MC3T3 cell viability and cell proliferation rates when compared with lower PEGDA concentration-containing gels. Gels containing higher concentrations of PEGDA also released both BSA

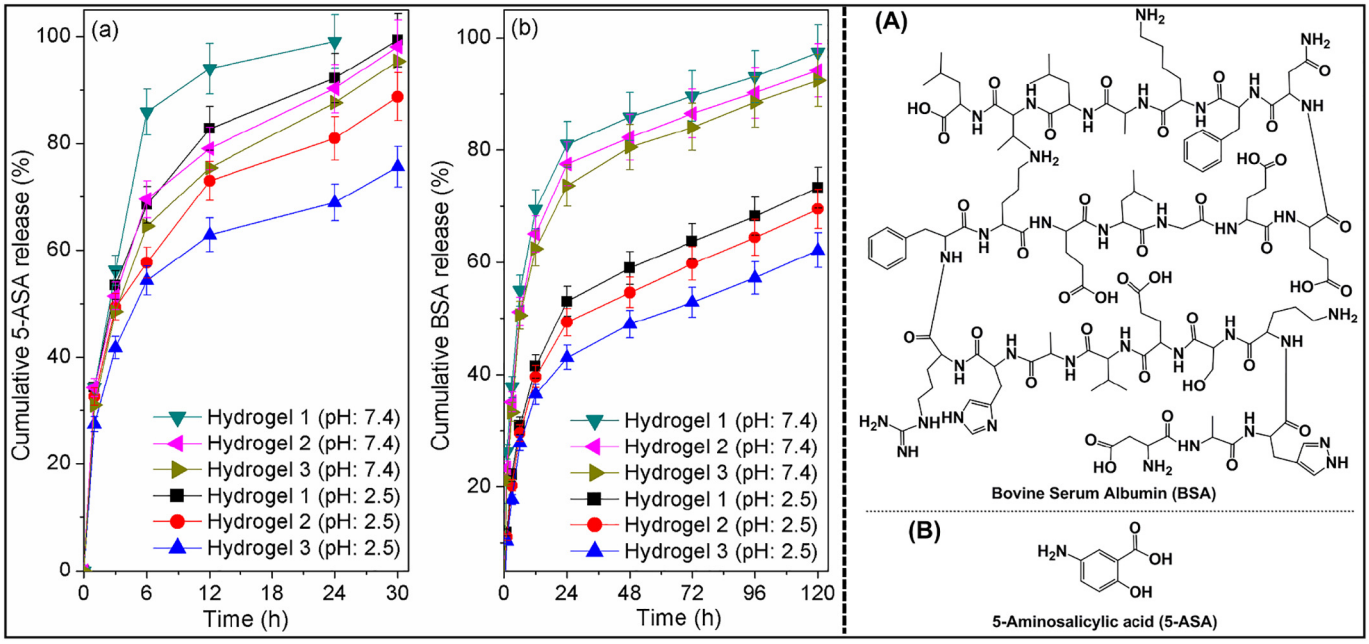


Fig. 10. *In vitro* release profiles of (a) 5-ASA and (b) BSA from three terpolymeric semi-IPN hydrogels at pH 2.5/7.4 and 37 °C, and chemical structures of (A) BSA and (B) 5-ASA.

and 5-ASA in more sustained way, whereas release rates were faster at pH 7.4 than that at pH 2.5. The release nature of 5-ASA and BSA throughout > 30 h, and 5 days signifies that the synthesized gel could be an efficient controlled release matrix for both 5-ASA and BSA. The experimental results revealed that the pH-responsive, and biocompatible semi-IPN hydrogel could be employed in biomedical applications especially for protein (bovine serum albumin) and colon-targeted drug (5-amino salicylic acid) delivery.

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