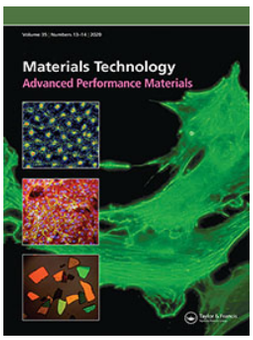

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3-Sulfopropyl methacrylate based cryogels as potential tissue engineering scaffolds

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ABSTRACT

In this study, we developed cryogels containing 3-sulfopropyl methacrylate (SPMA) and 4-vinyl pyridine (4-VP) as a potential scaffold for tissue engineering applications. Cryogels with varying monomer ratios were synthesised by chemical cross-linking under cryogelation conditions. Effect of initiators and cross-linker amount (0.025–0.15 g MBA; 0.012–0.05 g APS; 2.5–12.5 µl TEMED) and also freezing temperature (−20 and −80°C) were investigated, and the conditions were optimised according to the morphological structures examined by SEM. The functional groups of the materials were characterised by FT-IR. Compression test and swelling were applied to investigate mechanical properties and water absorption ability, respectively. As a preliminary study, selected materials were tested for cell cytotoxicity with MTT. According to our results, the ionic and biocompatible cryogels prepared in this study possessing a highly porous and interconnective structure with good mechanical characteristics and swelling properties can be suitable as tissue scaffolds for many applications.

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Cryogel; ionic; porosity; 3-sulfopropyl methacrylate

Introduction

Due to their extraordinary properties, cryogels, macroporous materials with high toughness, and fast responsivity have been attracted very high interest for the last two decades [1–4]. In the cryogelation process, the reaction mixture is cooled below the freezing point of the system. The solvent crystals, which are enriched by the monomers and the initiator, surround the unfrozen regions where polymerisation reaction takes place. Solvent crystals act as porogen and form a macroporous structure in the final material. Here, removal of the porogen material or template is achieved by applying heat above the solvent freezing point, which is one of the essential advantages of the cryogelation system over the phase separation technique in which the time-consuming extraction process is applied [5].

Cryogels have been investigated as tissue engineering scaffolds in different application areas such as wound healing, neural, bone, cardiac muscle, cornea, and cartilage tissue, as reported in many studies [6–13]. For cryogel preparation, poly(ethylene glycol) is the most widely used polymer due to its ability to show considerable swelling and mechanical characteristics as well as high biocompatibility.

Several studies reported the cryogel formation with different polymers such as PEG, gelatin, alginate, PLL. Sharma et al. mentioned that PEG–gelatin cryogel matrices display excellent mechanical properties and provide controlled cellular architecture [14]. Another study by Hwang et al. investigated the role of polymerisation rate on poly(ethylene glycol) (PEG) cryogel formation controlled by TEMED. They also studied the effect of freezing temperature on the gelation process [15]. Tripathi et al. reported that using gelatin increased the elasticity, cell adhesion, and proliferation in the agarose cryogel scaffold [16]. Bone and cartilage tissues are other broad application areas for cryogels that can be modified by additives to enhance applicability and repair abilities. Mishra and his friends showed that biocomposite cryogels affect the tissue regeneration significantly for the critical size bone defects, and osteoblastic differentiation in Wistar rats over four weeks [17]. In another study, HA-gelatin-based cryogels were produced and physicochemically characterised for the potential application in bone regeneration [18]. Min-Eui Han et al. investigated the potential of gelatin-based cryogel scaffolds for cartilage tissue engineering using primary rabbit chondrocytes and discussed the porosity and morphological characteristics [19].

As reported in many studies, cross-linking has been applied to achieve enhanced stability and excellent mechanical characteristics for the materials fabricated by freeze-drying or solvent-casting, as well as cryogelation. For example, Liu et al. reported the fabrication of silk fibroin porous materials by freeze-drying followed by cross-linking with PEG-diglycidyl ether. According to the results, they achieved appropriate mechanical characteristics and anticoagulant properties that can be advantageous as vascular autografts [20]. In another report, hybrid PCL-collagen scaffolds

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with different collagen concentrations were prepared by solvent casting and freeze-drying techniques with a subsequent chemical cross-linking. These materials having highly interconnected porosity, were investigated for tendon regeneration [21]. Ebrahimi and colleagues studied chemically cross-linked silk nanofibrous materials as a nerve guidance material. They achieved good resilience and compliance by chemical cross-linking and reported that significant cell adhesion and growth were obtained as a potential material for nerve tissue regeneration [22]. By the motivation from these studies, we also applied chemical cross-linking during the cryogelation process.

Lin Sun and colleagues reported that polyampholytes, polymers having cationic and anionic monomeric subunits dispersed randomly, form hydrogels with remarkable mechanical properties. They mentioned that the randomly distributed charges cause multiple ionic bonds with a wide distribution of strengths, by inter and intrachain complexation. In their report, it was concluded that prepared hydrogels with positively and negatively charged groups along the chain showed excellent biocompatibility and non-fouling properties, and these can be utilised as scaffold materials [23]. Zurick and colleagues prepared polyampholyte hydrogels having different charged monomers for potential use as tissue engineering scaffolds. They stated that physical characteristics of such hydrogels could easily be tuned that allow reaching ideal characteristics for a scaffold. In their report, they prepared a polyamholyte hydrogel from charged 2-carboxy ethyl acrylate and SPMA and investigated the physical properties, non-fouling properties, and conjugation capability [24]. They concluded that these ionic hydrogels having different charged monomers possess interesting physical properties while retaining their non-fouling characteristics, compared to the conventional hydrogels. In our study, we have used two oppositely charged monomers to obtain a putative scaffold material with good swelling, mechanical and biocompatibility characteristics. We also added cryogelation and cross-linking to the process in order to achieve a cryogel possessing all advantages of existing hydrogel and cryogel systems. Ionic polymers, including zwitterionic ones, have gained increased attention as they possess similarities with PEG in terms of hydrophilicity and biocompatibility. In recent studies, ionic polymers have been used as protective shells for micelles as well as improving the antifouling properties and stability of coatings [25]. According to Lucas et al.'s study, in copolymers containing ionic or sulphonated groups, the extent of foreign body reaction is significantly decreased [26]. Mai et al. reported that anionic monomer (SPMA) containing brushes showed excellent efficiency as mineralisation templates for calcium phosphate formation [27]. In another study, SPMA based brushes were applied as a cell-repulsive coating and studied in a neural guidance system combined with a micropatterning technique [28]. Based on the recent biomaterial-related applications, it is worth to investigate the characteristics of cryogels made of ionic monomers for potential scaffold applications. Cryogels prepared from PEG or ionic monomers, or many other comonomers have also been studied to change the chemistry and morphology of the final products. 4-vinyl pyridine is one of those monomers due to its protonation and quaternization abilities providing different application areas such as drug or DNA carriers and wound dressing materials [29–31].

According to the many cryogel preparation studies, the effect of monomer concentration, cross-linking degree, solvent type, and cooling temperature have been reported with different copolymer systems having varying monomer compositions. Here, we studied the cryogelation of SPMA with 4-VP for the first time in literature and reported the effect of different parameters on the process, and hence the resulted structure. The novelty of our study comes from using oppositely charged units in polymeric structure, the combination of cryogelation, as well as cross-linking, to reach new characteristics for a putative scaffold material. The porosity and interconnective nature of these materials have been evaluated together with the swelling abilities. We also confirmed the well-known advantages of cryogelation over hydrogel systems with our system and concluded that the materials have a great potential to be used as tissue engineering scaffolds.

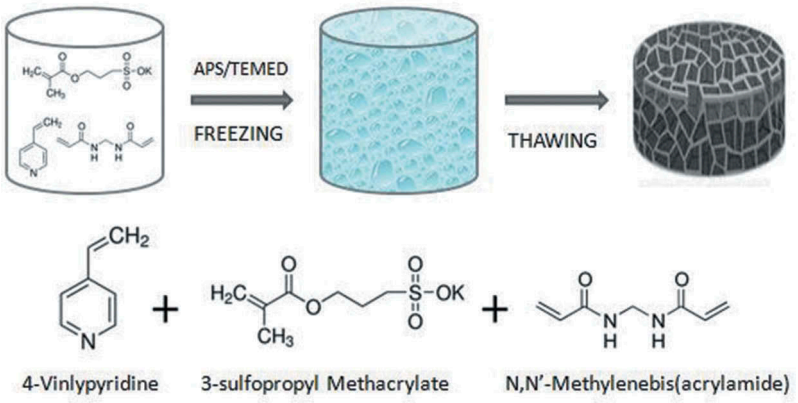
Materials and methods

Materials

SPMA was purchased from Sigma-Aldrich. N,N′-methylene bis(acrylamide (MBA)), N,N,N′,N′-Tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich. Ammonium persulphate (APS) was purchased from Ampresco (USA). Cell toxicity and proliferation were analysed by CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA). DMEM and PBS were purchased from Biological Industries (USA). FBS (Foetal Bovine Serum) was purchased from Gibco (Thermo Fisher Scientific, USA). All other reagents were in analytical grade and used as received.

Synthesis of the 4-VP/SPMA based cryogels

Scheme 1 is an illustration of the cryogel synthesis process. MBA was dissolved in 2.5 ml distilled water mixed with other monomers (SPMA and 4-VP) at specific ratios (Table 1). Then, APS was added to the solution to initiate the polymerisation process.



Scheme 1. The fabrication process of SPMA and 4-VP-based cryogels.

Table 1. The compositions of the cryogels*.

Sample	MBA(g)	APS(g)	TEMED(µl)	4-VP(mol)	SPMA(mmol)
AY1	0.05	0.025	5	1	1.5
AY2	0.05	0.005	5	1	1.5
AY3	0.025	0.012	5	1	1.5
AY4	0.15	0.012	5	1	1.5
AY5	0.05	0.012	12.5	1	1.5
AY6	0.05	0.012	2.5	1	1.5
AY7	0.05	0.012	5	1	1.5
AY7S1	0.05	0.012	5	1	0.5
AY7S2	0.05	0.012	5	1	5
AY7S3	0.05	0.012	5	1	10
AY7V1	0.05	0.012	5	0.1	1.5
AY7V2	0.05	0.012	5	0.25	1.5
AY7V3	0.05	0.025	5	0.5	1.5
AY7V4	0.05	0.025	5	0.75	1.5

*Temperature: -20°C.

Following that, the TEMED was added, and the mixture was transferred into the moulds. They were frozen overnight for the cryogelation process, and diethyl ether was added for the solvent exchange process at −20°C for 24 h. Finally, the syringes were cut as discs and washed with distilled water, thoroughly.

Characterisation

Morphology

Scanning electron microscopy (SEM, ZEISS LS-10) was utilised for the investigation of the pore structure and interconnectivity of the SPMA cryogels. The samples were first lyophilised for 24 h and coated with gold by using QUORUM, Q150R ES coater.

Swelling characteristics

The water absorbtion ability of each cryogels was determined by measuring swelling ratios. Each cryogels were immersed in distilled water at room temperature for 24h. Then the cryogels were weighed and the equilibrium swelling ratios of the samples were determined by the following equation.

$$S(\%) = \frac{Mw - Md}{Md} \times 100$$

where Mw is the weight of wet cryogel, Md is the weight of dried cryogel.

FT-IR analysis

Thermo Scientific Nicolet 6700 FT-IR Spectrometer (USA) was utilised for the FT-IR analysis of the cryogels in the range of 400–4000 cm^{-1}.

Mechanical test

Before the compression test, the cryogels were immersed in distilled water for 15 min to achieve equilibrium swelling. Compression tests were performed using a tension-compression test machine (Shimadzu AG-XD 50kN). Samples were compressed with two parallel plates at the maximum loading of 100 N with a compression rate of 1 mm/min. The stress, strain (%), and toughness values were calculated by the Trapezium X Materials Testing Software (Shimadzu, Japan). The compressive modulus was calculated from the linear region of the stress-strain curve (0–10% strain). A hydrogel was synthesised at the same conditions (without freezing) for comparison.

Cytotoxicity

Because of their potential application area is tissue engineering, we performed cytotoxicity towards fibroblast cells. First, ethanol-sterilised cryogels were cut and placed into the 96 well plates. In DMEM containing %10 FBS and %1 penicillin/streptomycin at 37°C, the cells were seeded onto the cryogels at a density of 1×10^5 cells/mL, and they were incubated at 37°C for 48h. 20µl of CellTiter 96® AQueous One Solution Reagent was added into each well, and the plate was incubated at 37°C for 24 h. At last, the absorbance at 490 nm was recorded. All data are presented as mean standard deviation (SD). A single-factor analysis of variance (ANOVA) was performed to determine statistical significance ($p < 0.05$).

Results and discussion

Cryogel synthesis and characterisation

SPMA and 4-VP-based cryogels were synthesised by radical polymerisation using redox initiators APS/TEMED and cross-linking in the presence of the MBA. We investigated the effect of MBA, TEMED, and APS and temperature on cryogel formation by changing concentrations and freezing conditions (Table 1). Cryogel preparation was performed at varying freezing temperatures as −20 and −80°C because it plays a critical role in network structure formation. The best cryogels were obtained at −20°C, so we continued with this temperature for the synthesis of all samples.

FT-IR spectra of the copolymer cryogels are characterised by the typical absorption bands for 4-VP units (C-N stretching at 1200 cm^{-1} and ring stretching at 1600 cm^{-1}) and SPMA units (carbonyl ester stretching peak at 1720 cm^{-1}, C = C bending at 980 cm^{-1}). The addition of 4-VP to the cryogel structure was confirmed by the change in intensity of the peaks observed at 1200 cm^{-1} and 1600 cm^{-1} (Figure 1). The intensity of the peak appeared at 1720 cm^{-1} increased by the change of SPMA amount (0.5, 5, and 10 mol) in cryogel structure (Figure 2). FT-IR spectra show the

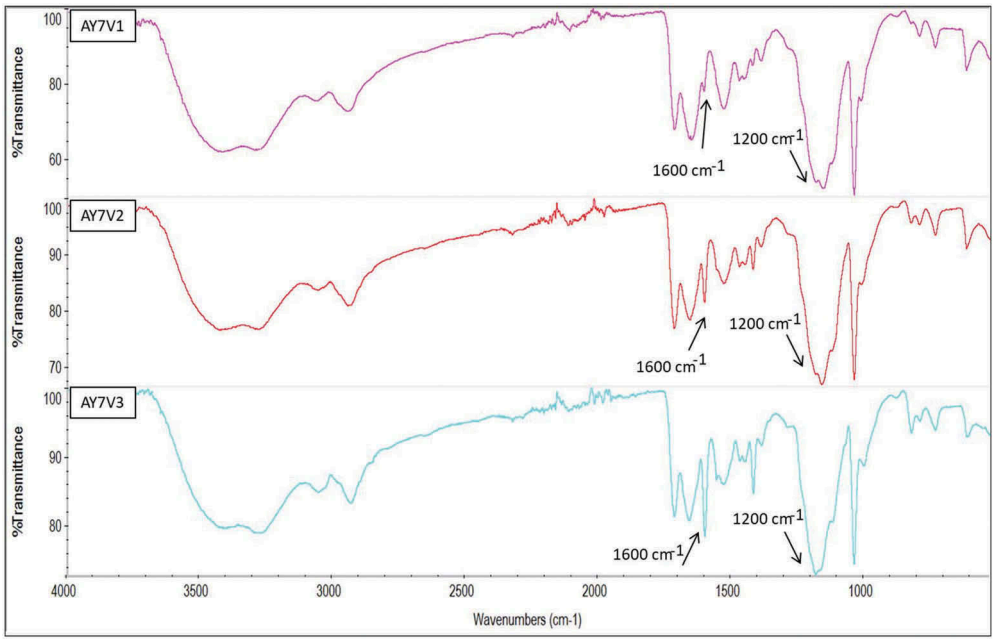


Figure 1. FT-IR spectra of cryogels with different 4-VP amounts (AY7V1, 2, 3).

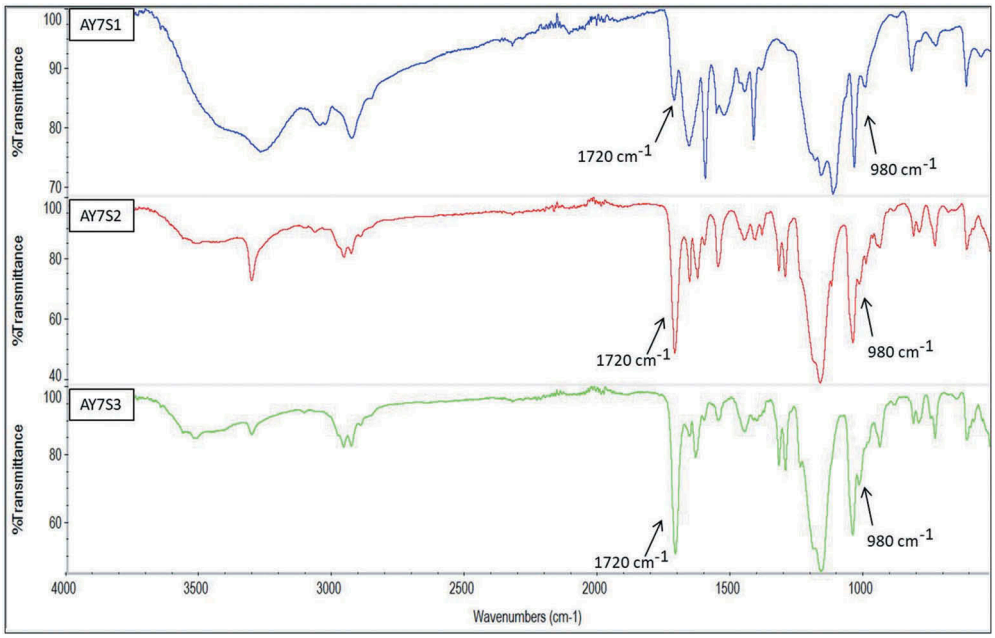


Figure 2. FT-IR spectra of cryogels with different SPMA amounts (AY7S1, 2, 3).

cryogels containing different 4-VP and SPMA contents were synthesised successfully.

Morphology and swelling

Porosity and interconnected pore structure are crucial for an ideal tissue engineering scaffold independent from the type of tissue. Here, we have changed many different parameters, such as the concentration of monomer, initiator, cross-linker, and cryogelation temperature. All of these parameters caused differences in morphology and pore structure as well as swelling behaviour.

For cryogelation temperature optimisation, we have applied −20 and −80°C for the freezing process and obtained better porosity at −20°C (Figure 3). According to the Okay and Lozinsky, at lower cryogel preparation temperature, since the freezing rate is increased, a high number of small solvent crystals form [5]. Also, a freezing temperature close to the solvent freezing point leads to larger pores because the solvent in large voids freezes during the gelation process. Therefore, all other experiments were conducted at lower freezing temperature (−20°C).

Swelling experiments were conducted in distilled water, as mentioned in the method part, and the swelling ratio was calculated depending on time, according to Equation 1. Figure 6 shows the swelling behaviour of each cryogel depending on time. We also investigated the effect of redox initiators and other ingredients on swelling and evaluated together with porosities examined by SEM (Figures 4 and 5).

First, the effect of the TEMED amount on cryogelation process and polymerisation rate, and also network structure was investigated. Changing TEMED quantity in polymerisation medium (12.5, 2.5, 5 µl as AY5, 6, 7), which affects the polymerisation rate, resulted in cryogels with different microstructures [12]. Materials showed a homogenous network structure, which is seen from their morphological appearance and swelling behaviour. According to the SEM images (Figure 4), they have an interconnected macroporous structure with a pore size between 50–100 µm. By the increase in the TEMED amount, larger pores are obtained but the structure was more homogeneous at lower TEMED concentrations, which is probably caused by the competition between the rate of gelation and the rate of ice crystal nucleation. Hwang and Varghese's study reported that the microstructures of cryogels are dependent on the kinetics of these two processes [12]. In a heterogeneous, the formation of hydrogel-like regions is faster than the ice crystal nucleation process. Swelling data also confirmed this observation that higher swelling ratio was achieved as the TEMED concentration decrease (Figure 6).

MBA effect was investigated by changing MBA as 0.025, 0.05, 0.15 gr (AY3, 7, 4). While it was decreased, larger pore sizes with an interconnective nature were achieved compared to AY4 and AY7. Also, AY4, which has the highest MBA amount, showed a deteriorated pore structure because of the very high cross-linking process. AY3 has the highest swelling ratio because of homogeneous and uniform pore structure, probably. The lowest swelling ratio can be another confirmation for the deteriorated pore

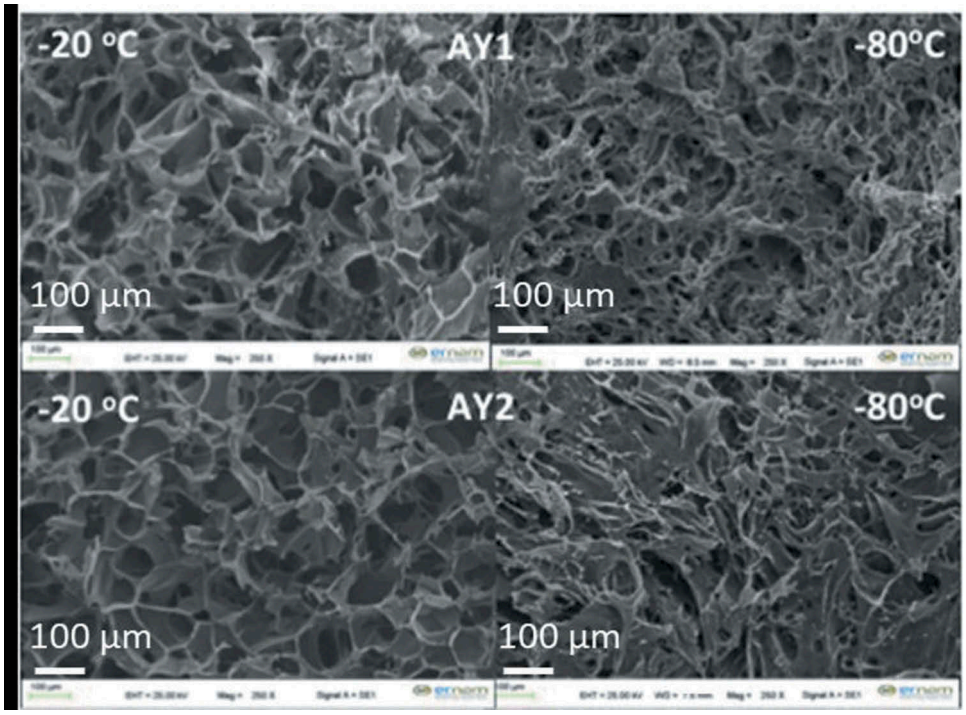


Figure 3. Effect of cooling temperature (−20 and −80°C) on cryogel morphology.

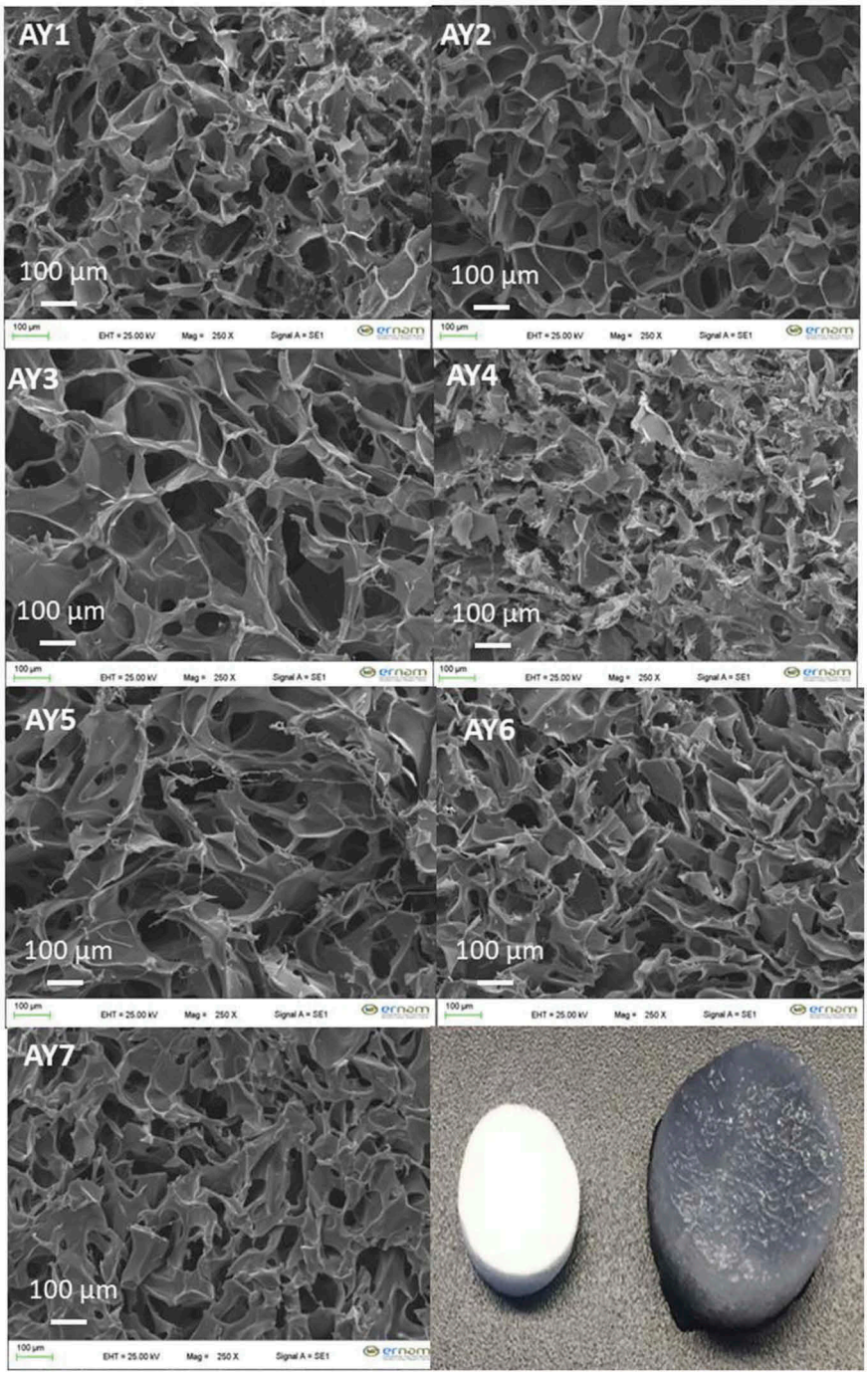


Figure 4. SEM images of cryogels with varying APS, TEMED and MBS concentrations (AY1-7).

structure of AY4 (Figure 6). As the amount of cross-linker is increased, the rigidity of the gel in the unfrozen domains is also increased [5]. Changing the APS amount did not cause any dramatical difference between pore structures, but the best porosity observed in AY2, as seen in Figure 4. An increase in APS decreases the pore size, here we have observed a kind of smaller pore size with AY1 at which the lowest APS amount [32].

The addition of SPMA to the cryogel structure lowered the swelling ability of the cryogels (AY7S1, 2, 3 for 0.5, 5, 10 mmol SPMA, respectively) as also confirmed by pore structure. As seen in Figure 5, the difference between the porosities of AY7S1 and AY7S3 was noticeable that reflects the swelling characteristics as well (Figure 6). According to the Okay et al.'s report, the pore size decreased by the increase of monomer concentration [5].

The cryogels obtained at varying 4-VP ratios cryogels (AY7V1, 2, 3 for 0.1, 0.25, 0.75 mol 4-VP, respectively) showed suitable pore structures which have homogeneous and interconnected (Figure 5). Only the low amount of 4-VP caused smaller pores. Swelling ratios of these three cryogels with varying 4-VP amounts were lower than all other cryogels because the 4-VP amount was kept as 1 mol. The effect of monomer concentration was also confirmed by this data [5].

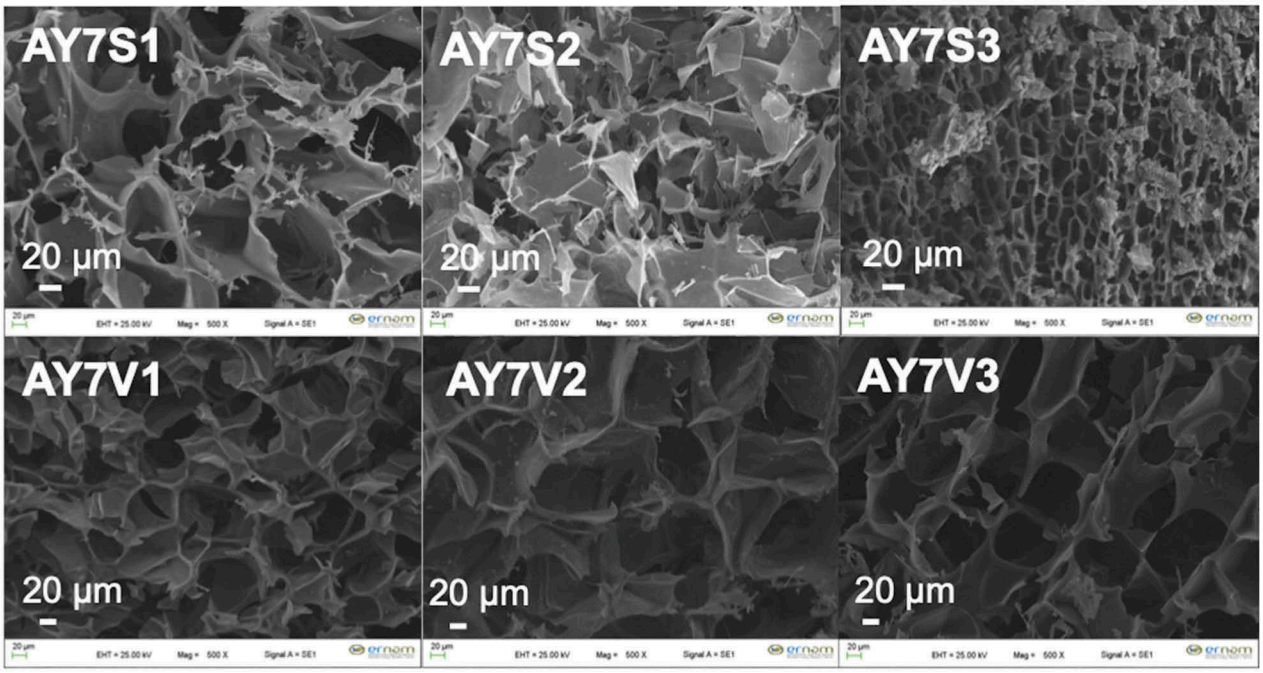


Figure 5. SEM images of cryogels with varying SPMA and 4-VP concentrations (AY7S1-3 and AY7V1-3).

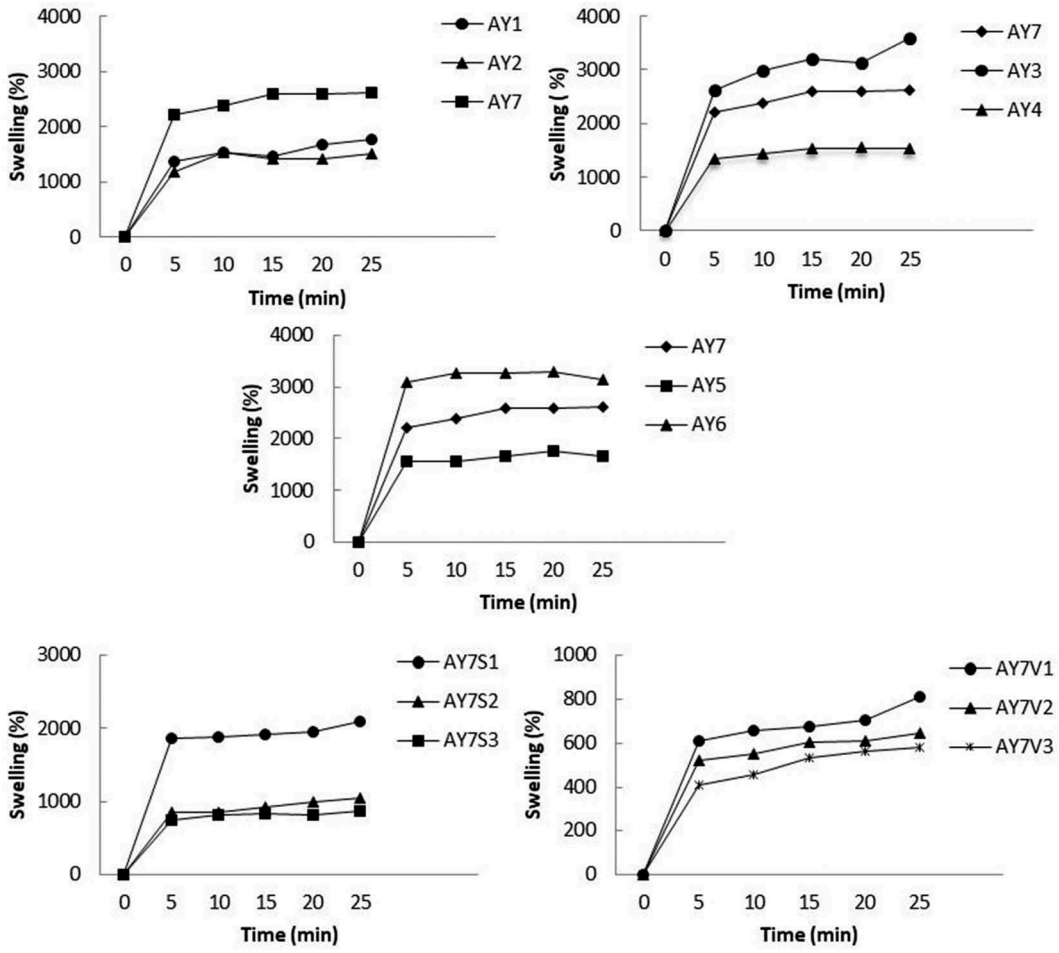


Figure 6. Swelling behaviour of different cryogels (AY1-7, AY7S1-3, and AY7V1-4).

According to the all data, swelling abilities of cryogels here are dependent on different parameters but in quite high levels compared to the similar cryogel systems. In Maitlo et al.'s study, cross-linked cryogels containing HEA, HEMA and PEGDA showed up to 450% water absorption [33]. We achieved better swelling and porosity caharcteristics most likely due to ionic monomer nature through the polymeric structure.

Mechanical test

Cryogels should have adequate strength to be used as a scaffold. Therefore, we performed the required tests to prove its mechanical performance. Note that, we prepared a hydrogel at the same conditions without applying freezing to make a comparison in terms of mechanical behaviour.

Compared to the hydrogel, cryogels exhibited lower compressive modulus (Table 2). The hydrogel showed 18.16% deformation and 1.10 MPa of maximum stress, thereby exhibiting brittle mechanical behaviour. The compressive modulus of hydrogel confirmed brittleness of the material, and it was reported in Hwang et al.'s study in which increasing of the compressive modulus indicated the brittle behaviour [15]. In contrast, the cryogels showed more significant deformations and lower fracture stress than the material in hydrogel form (Table 2). The AY7 cryogel exhibited a 27.09% deformation and fracture stress of 0.79 MPa with the lowest compressive modulus. According to the literature, the lower compressive modulus was explained by elastic behaviour [15]. AY1 (−80°C) showed less deformation than AY1 (−20°C) (AY1 28.84%, AY1(−80°C) 16.94%) meaning that the sample prepared at −20°C has more elastic nature with cryogel characteristics. Note that, according to the morphological analysis, the cryogel sample at −20°C had better porosity than the one prepared at −80°C that might support the mechanical test result here (Figure 3). We did not observe any dramatic difference between the AY1 and AY6, mechanically, which was also supported by the SEM and swelling data. To understand the effect of monomer concentration on mechanical properties, we compared AY7S1 and AY7S3 at which SPMA contents were changed as 0,5 and 10 mmol, respectively. Higher deformation was observed with AY7S3 having a denser cryogel structure and lower swelling [5–13]. It can also be concluded that AY7S3 has more elasticity than the AY7S1 due to smaller compressive modulus. To summarise, ionic cryogels prepared here showed durable mechanical characteristics which has a great importance in scaffold applications. This was also confirmed by Bhat et al.'s report in which durable mechanical and tensile properties has a primary importance in cartilage tissue engineering [34].

Table 2. The compression test result of selected cryogels.

Sample	Stress at break (MPa)	Strain at break (%)	Toughness (kJ m^{-3})	Compressive modulus (MPa)
AY7	0.79	27.09	20.78	0.068
AY1	0.89	28.84	33.99	0.195
AY1(−80 °C)	0.51	16.94	13.01	0.54
Hydrogel	1.10	18.56	22.91	0.62
AY7S1	0.90	17.40	10.8	0.48
AY7S3	0.78	41.59	43.15	0.30

In vitro cell proliferation and scaffold cytotoxicity studies

SPMA based cryogels (AY7S1-3) were analysed using fibroblast cell lines to observe in vitro cell proliferation and biocompatibility by CellTiter 96® Aqueous One Solution Reagent. The analysis quantified by checking the optical density via spectrophotometric analysis. Figure 7 shows the results of the cell viability test at 48 hours, and according to the results, all cryogels are biocompatible and suitable for cell growth. Increasing the SPMA concentrations affect cell viability positively, which confirms its biocompatibility due to its ionic character.

Conclusion

In this study, we aimed to prepare cryogels containing SPMA and 4-VP, charged monomer units, for potential tissue engineering scaffold applications. FT-IR results confirmed polymerisation, and SEM images approved the effect of APS, TEMED, and MBA on morphology. Quite high (up to 3500%) swelling ratios were achieved with the cryogels, and these results were in consistency with the pore structures. They revealed suitable mechanical characteristics, which are also compatible with the morphological and swelling behaviour. At last, the cytotoxicity test showed that the addition of SPMA to the cryogels increased biocompatibility. According to the results, these novel ionic cryogels having oppositely charged monomer units have a significant potential to be used as a scaffold for a specific tissue in the body.

Disclosure statement

No potential conflict of interest was reported by the authors.

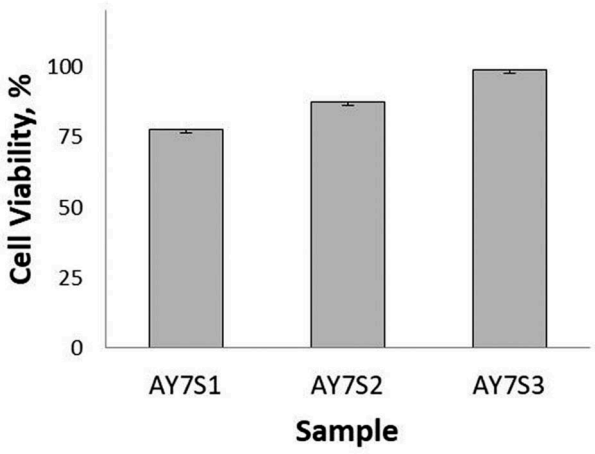


Figure 7. Cytotoxicity of cryogels containing different SPMA amount on fibroblast cells.

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