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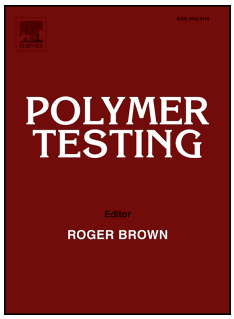

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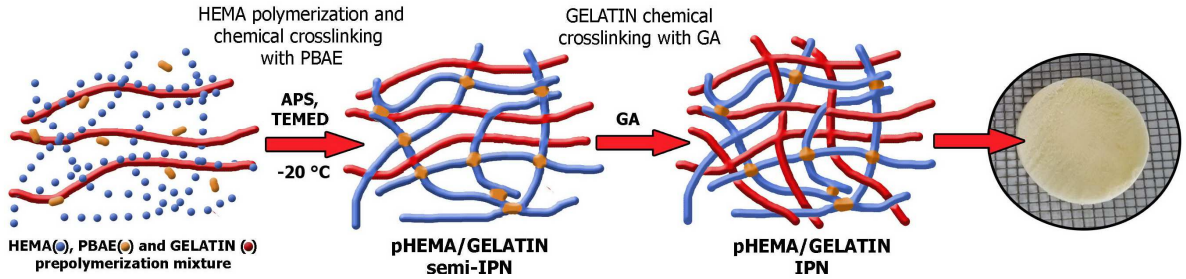

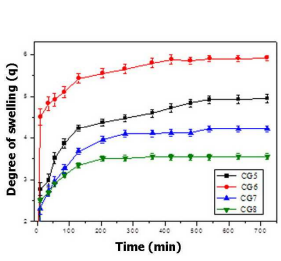

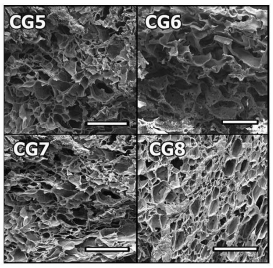

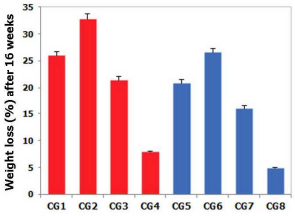

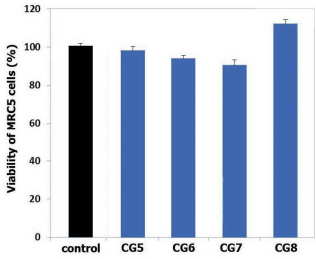

Biocompatible and degradable scaffolds based on 2-hydroxyethyl methacrylate, gelatin and poly(beta amino ester) crosslinkers

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ABSTRACT

Gelatin hydrogels have great potential in regenerative medicine but their weak mechanical properties are a major drawback for the load-bearing applications, such as scaffolds for tissue engineering. To overcome this deficiency, novel biodegradable hydrogels with improved mechanical properties were prepared by combining gelatine with 2-hydroxyethyl methacrylate (HEMA), using a double network synthetic procedure. The first, superporous and mechanically strong network, was obtained by free radical polymerization of HEMA at cryogenic temperature, in the presence of gelatin. Degradable poly(β-amino ester) (PBAE) macromers of different chemical composition or molecular weight were used as crosslinkers to introduce hydrolytically labile bonds in PHEMA. The second gelatin network was formed by crosslinking gelatin with glutaraldehyde. For comparison, a set of biodegradable PHEMA networks was obtained by polymerization of HEMA at cryogenic temperature. All samples were characterized revealing that mechanical strength, swelling behavior and degradation rate as well as high biocompatibility of new IPNs are in accordance with values required for scaffolds in tissue engineering applications and that tuning of these properties is accomplished by simply using different PBAE macromers.

1. INTRODUCTION

Hydrogels are slightly crosslinked hydrophilic polymers that swell and can hold varying amounts of water, but do not dissolve. Their rapidly increasing use in biomedical applications, such as drug delivery and tissue engineering biomaterials, is due to their consistency, high water content and soft texture, which is very similar to natural living soft tissues. Besides, there is a possibility to tune the hydrogel biocompatibility, chemical and physical properties for the targeted applications using different components and synthetic procedures. In recent years an increasing interest for highly porous biodegradable polymeric hydrogels arose in the development of advanced tissue engineering materials, such as implants and scaffolds in tissue engineering. Biodegradability is required in order to avoid the need for second surgery [1]. The conjugation of biological and synthetic materials, which are similar to the extracellular matrix (ECM) components, is a good way to produce the hydrogel scaffolds that mimic natural ECM for the tissue engineering and regenerative medicine applications [2, 3]. In this respect biodegradable components, as gelatin and HEMA, can be considered as a good combination for hybrid hydrogel scaffolds in tissue regeneration, as they consolidate the benefits of a natural and synthetic polymer. Gelatin, as an enzymatically biodegradable polymer which introduces biological cues in the scaffold, is reinforced by PHEMA hydrogel, crosslinked with hydrolytically degradable crosslinkers. Synthetic polymers, such as PHEMA, also allow tuning of physical and chemical properties of hybrid hydrogel [4].

Gelatin gained much attention as scaffold material due to excellent biocompatibility, biodegradability, low immunogenicity, availability, easy handling and low cost [5]. PHEMA is a synthetic polymer which is nontoxic and has good biocompatibility, chemical stability, mechanical properties and elasticity, as well as a relatively high degree of hydration, but it is not biodegradable and cannot be eliminated from the body [6]. There are several examples in the literature of introducing labile linkages in HEMA to obtain biodegradability [7, 8]. Huang et al [7] designed degradable, elastomeric PHEMA/hydroxyapatite biocomposites using a degradable crosslinker (macromer) dimethacrylated triblock copolymers, poly(lactide-b-ethylene glycol-b-lactide) (PLA-b-PEG-b-PLA) which disintegrate by hydrolytic degradation [9, 10]. Moghadam et al [8] used N,O-dimethacryloyl hydroxylamine (DMHA), sensitive to hydrolytic cleavage, to fabricate biodegradable PHEMA hydrogels. The use of synthetic PBAE macromers as degradable crosslinkers was only investigated for the synthesis of P(HEMA/NVP) copolymers by R A. McBath and D. A. Shipp, but they investigated only the swelling and degradation properties and did not suggest any application of these hydrogels [11].

PBAEs display numerous advantages, including good biocompatibility, simple synthetic procedure, and pH and temperature sensitive swelling and degradation [12, 13]. A wide range of different, structuraly diverse PBAEs can easily be aquired by changing either the reacting amine or diacrylate

component, or their ratio. PBAEs have a large variety of applications including encapsulation and delivery of biological molecules, as nanocarriers for mRNA [14] and DNA [15], antioxidans [16] and drugs for cancer [17] and cartilage treatments [18]. Although synthetic PBAE macromers found numerous applications in medicine and pharmacy, their use was focused on the synthesis of PBAE polymers and copolymers [19-20]).

In recent years biodegradable macroporous polymeric hydrogels have become highly important in the field of biomaterials and tissue engineering. They have attracted much attention, particularly in applications such as 3D scaffolds for the repair and regeneration of injured tissue, as templates for tissue regeneration which guide the growth of new tissue. A scaffold should have an open-cell structure with different pore sizes, improving both cell proliferation and vascularization and also providing the exchange of liquids and nutrients for cell survival [21]. Due to biodegradable property a need for additional surgeries to remove the implants or scaffold is avoided [22].

The novelty of this work is that gelatin and HEMA, crosslinked with PBAE macromers, were used for the first time to prepare scaffolds for tissue engineering applications, as a combination of natural and synthetic polymer which is producing new material with tailored functional properties. The double network sequential synthetic procedure at cryogenic conditions was used to obtain the superporous IPNs with interconnected pores, as a uniform mixture of these polymers. The second set of biodegradable simple networks was obtained under the same conditions, by free radical polymerization of HEMA crosslinked with PBAE macromers. The aim of this study was to tune the degradation and mechanical properties of IPNs, by simply changing the type and/or molecular weight of PBAE macromer. The characterization of samples for their chemical composition, morphology, porosity, swelling, and mechanical properties as well as for degradation behaviour and cytotoxicity studies confirmed that PHEMA/gelatin IPNs would be suitable for potential application as scaffolds for tissue engineering.

2. MATERIALS AND METHODS

2.1. Materials

Piperazine (PIPz, 99%), HEMA, di(ethylene glycol) diacrylate (DEGDA), 1,6-hexanediol diacrylate (HDDA) and gelatin (porcine, type A) were purchased from Aldrich. Poly(ethylene glycol) diacrylate ((PEGDA) average Mn 400) was purchased from Polysciences (Hirschberg, Germany). Potassium persulfate (KPS, Aldrich), as initiator, and *N*,*N*,*N′*,*N′*-tetramethylethylene diamine (TEMED, Aldrich), as activator, were used in all polymerizations performed in cryogenic conditions at - 20°C. Potassium hydrogen phosphates (KH_2PO_4 and K_2HPO_4, Aldrich) were used

for buffer preparations. Deionized water was used for all polymerizations and the preparation of buffer solution.

2.2. PBAE macromer synthesis

PBAE (**CL1** and **CL2**) macromers (Scheme 1) were synthetized by Michael addition of DEGDA and piperazine, according to the previously described procedure [11]. PBAE macromers were used without any further purification.

PBAE macromer **CL1** was synthesized by vigorous stirring of DEGDA solution (3 mL in 3mL $CHCl_3$) at 25°C, to which was added piperazine solution (0.622 g in 3 mL $CHCl_3$) in 3 equivalent portions, at 0, 12 and 24 h. The molar ratio of diacrylate to piperazine was 1.8/1. The stirring was continued for additional 24 h. The solvent was then evaporated from the reaction mixture, leaving a yellow viscous fluid.

Sheme 1. Synthesis of **CL1** and **CL2** macromers.

PBAE macromer **CL2** was synthesized by vigorous stirring of DEGDA solution (10 mL in 5 mL $CHCl_3$) at 25°C, with adding piperazine solution (3.685 g in 5 mL $CHCl_3$). The molar ratio between diacrylate and piperazine was 1.2/1. The stirring was continued for additional 6 h. The solvent was then evaporated from the reaction mixture, leaving a dark yellow viscous fluid.

PBAE macromer **CL3** (Scheme 2) was synthesized by stirring 1,6-hexanedioldiacrylate solution (7.30 g, 0.0259 mol, in 6 mL $CHCl_3$) at 50°C, and adding piperazine solution (1.24 g, 0.0144 mol, in 6 mL $CHCl_3$) in 3 equivalent portions, at 0, 12 and 24 h. The molar ratio of diacrylate to piperazine was 1.8/1. The stirring at 50°C was continued for additional 24 h. The solvent was then evaporated from the reaction mixture, leaving a pale yellow viscous fluid.

Sheme 2. Synthesis of **CL3** macromer.

2.2. PBAE characterization

Chemical structure of poly (beta amino) esters was determined using ^1H NMR spectra, recorded on a Bruker Ultrashield Advance III spectrometer (at 500 MHz), using CD_3Cl as solvent and TMS as the internal standard.

2.3. Cryogel syntesis (without gelatin)

The cryogels were obtained by free radical polymerization at cryogenic temperatures. HEMA (1.29 g, 9.8 mmol), PBAE macromers or PEGDA (15 % w/w for HEMA) were dissolved in 5 mL of

deionized water, using APS as initiator and TEMED as activator. The reaction mixture was purged with nitrogen for 10 minutes, poured into petri dish (5 cm radius, 0.5 cm height) and cooled to -20 °C for 24 h. After thawing the cryogel at room temperature, it was cut into discs and washed with deionized water for 7 days in order to remove impurities. Water was changed daily. Swollen cryogels were frozen and freeze-dried.

2.4. Cryogel synthesis (with gelatin)

PHEMA/gelatin cryogels were obtained by free radical polymerization, using APS as initiator and TEMED as activator. HEMA (1.29 g, 9.8 mmol), PBAE macromers or PEGDA (15 % w/w for HEMA) and gelatin (300 mg) were dissolved in 5 mL of deionized water, by stirring at 40°C. This mixture was purged with nitrogen for 15 min, poured into petri dish (5 cm radius, 0.5 cm height) and cooled to -20°C for 24 h. After thawing the cryogel at room temperature, it was cut into discs which were submerged in 4% glutaraldehyde solution overnight, in order to crosslink gelatin (Fig. 1). Cryogel samples were washed with deionized water for 7 days to remove impurities. Water was changed daily. Swollen cryogels were frozen and freeze-dried. The cryogels were designated as in a following table (Table 1).

Fig. 1. Semi-IPN and IPN synthesis.

Table 1. The composition and designation of cryogels.

Cryogel	Monomer(s)	Crosslinker
CG1	HEMA	**CL1**
CG2	HEMA	**CL2**
CG3	HEMA	**CL3**
CG4	HEMA	**PEGDA**
CG5	HEMA, gelatin	**CL1**
CG6	HEMA, gelatin	**CL2**
CG7	HEMA, gelatin	**CL3**
CG8	HEMA, gelatin	**PEGDA**

2.5. Hydrogel characterization

Hydrogel composition was analysed using FTIR spectra, recorded on a Thermo-Scientific Nicolet 6700 FT-IR diamond crystal spectrometer, using the Attenuated total reflectance (ATR) sampling technique.

2.6. Swelling studies

Degree of swelling (q_e) for the cryogels was determined using the gravimetric method. Dry cryogel discs were immersed in buffer solution (phosphate buffer, pH 7.4) at 37 °C, mimicking physiological conditions, for 24 hours. Discs were periodically removed from the buffer solution, dried by blotting on paper to remove excess water, and weighted. Degree of swelling was calculated using the formula:

$$q_e = \frac{m_s - m_i}{m_i}$$

Where m_i is the initial weight of the dry cryogel, and m_s was the weight of the swollen cryogel at the time of measuring. Degree of swelling was plotted as a function of time.

2.7. Mechanical Testing

Mechanical characteristics of the scaffolds were measured with universal testing machine (Galdabini Quasar 50, Italy) by the application of a uniaxial compression with 100-N load cell at room temperature. The Young's modulus (E) was calculated from the linear part of the stress/strain curve and its final value is an average of three measurements.

2.8. Morphology

The morphological analysis was performed with scanning electron microscopy (Jeol JSM-7600F). Samples were cut into slices, fixed on holder using carbon tape, sputtered with gold (using BAL-TEC SCD 005) and dried in vacuum chamber (VC 50 SalvisLab Vacucenter). Observation was performed for cross sections of the scaffolds.

2.9. Porosity measurement

Porosity of cryogels was determined by solvent replacement method. Glycerol (ρ = 1,2038 g/cm^3) was used as a wetting medium. Dried cryogels were submerged in glycerol for 24 hours, and weighted after removing excess glycerol from the surface:

$$Porosity = \frac{(m_{glycerol} - m_i)}{\rho V} * 100$$

Where m_i is the initial weight of the dry cryogel, $m_{glycerol}$ is the weight of the cryogel with glycerol, ρ is the density of glycerol and V is the volume of the cryogel sample.

2.10. Degradation studies

In vitro hydrolytic degradation studies were conducted by submerging cryogel samples in phosphate buffer solution (pH 7.4 at 37 °C). Samples were taken out of the buffer solution at every two weeks, dried at 40°C until constant mass, and measured. Degradation was presented as a function of remaining cryogel mass percentage as a function of time.

$$Percent\ of\ remaining\ cryogel\ weight = \frac{m_t}{m_i} * 100$$

Where m_i is the initial weight of the dry cryogel, and m_t is the weight of the dried cryogel sample at the time of measuring.

2.11. Biocompatibility assay

To evaluate the biocompatibility of the novel cryogels is the first step in evaluation of newly synthesized molecules. For the biocompatibility assay in this study normal human fibroblast cells, MRC5, were used. The synthesized cryogels were cultivated in the cell culture media in 24-well microtiter plate during 24 hours. After this incubation period supernatants were centrifuged and used for biocompatibility assay. The MRC5 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were grown in culture bottles supplied with medium for cultivation, and thereafter cells were seeded in 96-well plate. MRC5 cells seeded in 96-well microtiter plate and cultivated in the full culture media in a humidified atmosphere of 5% CO2 at 37°C during 24 hours. The next 24 hours the MRC5 cells were incubated both with and without prepared supernatants of the investigated hydrogels. At the end of this period modified MTT method [23] was used for measuring the cell viability/proliferation. This test is based on the color reaction of mitochondrial dehydrogenase from living cells with MTT. Briefly, 10 μL of MTT solution (5 mg mL−1) was added to each well after cells treatment and cells were incubated for additional 3 hours at 37°C. Produced formazan was dissolved by overnight incubation with sodium dodecyl sulfate (SDS)-HCl mixture (10% SDS in 0.01 N HCl) and absorbance was measured at dual wavelength of 570/650 nm with an enzyme-linked immunosorbent assay (ELISA) 96-well plate reader. The percentage of viable cells was calculated as the ratio between absorbance at each dose of the compounds and absorbance of untreated control cells ×100.

3. RESULTS AND DISCUSSION

3.1. PBAE macromer synthesis

PBAE macromers, a class of biodegradable and biocompatible materials, were polymerized using the reaction of acrylates with amines, i.e. Michael addition, which proceeds via stepwise reaction. This reaction is easy to conduct and does not require any purification steps.

In this work different PBAEs macromers were synthesized using an addition reaction of the same amine component, piperazine, with two different diacrylates, di (ethylene glycol) diacrylate and 1,6-hexanediol diacrylate. As the diacrylate to diamine molar ratio was >1 in all samples, the final macromers had acrylate end groups, permiting free radical polymerization or copolymerization reaction and crosslinking of monomers, in order to prepare simple polymer networks [24]. Using diacrylates DEGDA and HDDA with different hydrophilic properties, different macromer hydrophilicity was obtained. As macromer hydrophilicity affects the swelling/degradation behavior, this property can be used to tune the degradation rate. The HDDA/piperazine ratio was 1.8/1. In order to obtain corresponding mechanical properties and degradation rates the DEGDA/piperazine molar ratio was varied (1.2/1 and 1.8/1). This afforded different molecular weights of macromers, providing variation in crosslinking densities and mechanical strength.

The ^{1}H NMR spectra of PBAE **CL1** confirmed that the synthetized PBAE macromers are pure and possess acrylate groups at the chain ends (Fig. 2). Determination of number-average molecular weight of PBAE macromers was achieved by comparing the integrated NMR data of two end group protons (vinyl protons of acrylate groups – 6 protons in 2 vinyl groups), with characteristic protons found in the repeating unit of the monomer (ethylene proton peaks of ethylene glycol in DEGDA and HDDA) [25].

Fig. 2. ^{1}H NMR spectra of CL1 macromer, with comparison of peak integral values.

By comparing the integral value of vinyl proton peaks (5.7 - 6.5 ppm) with those of ethylene proton peaks (3.5 - 4.4 ppm) from ethylene glycole fragment, for **CL1** and **CL2** and ethylene proton peaks (4.0-4.2 ppm) for **CL3**, the number-average molecular weight of poly beta amino ester macromers was calculated. Number-average molecular weights were as follows: 583 g/mol for **CL1**, 2944 g/mol for **CL2** and 603 g/mol for **CL3**.

3.2. Hydrogel synthesis

Two series of highly porous hydrogel networks, simple PHEMA and PHEMA/gelatin IPNs, were successfully synthesized.

3.3. Hydrogel characterization

Comparing the FTIR spectra of PBAE macromer **CL1** and cryogels **CG1** and **CG5** (Fig. 3), the absence of two characteristic acrylate bands from the **CL1** (C=C stretching at 812 cm^{-1} and 1635

cm^{-1}) can be observed in spectra of both cryogel samples [26]. This shows that vinyl end groups were converted into ethylene groups from PBAE macromers. Observable bands in **CG1** cryogel spectra are at – 3415 cm^{-1} for O-H stretching, 2944 cm^{-1} for C-H stretching of alkyl groups and 1722 cm^{-1} for C=O stretching of ester carbonyl group. In spectra of **CG5** cryogel observable bands are 3324 cm^{-1} for O-H and N-H stretching, 2944 cm^{-1} for C-H stretching of alkyl groups, 1722 cm^{-1} for C=O stretching of ester carbonyl group, 1653 cm^{-1} for C=O stretching of amide group (amide I), and 1549 cm^{-1} for N-H bending vibrations of amid group in gelatin (amide II) [27].

Fig. 3. Comparison between FTIR spectra of crosslinker CL1 and cryogels CG1 and CG5.

3.4. Swelling studies

Swelling capacity of degradable biomaterials is very important and closely connected with their degradation, because swelling is the first step in the hydrolytic degradation process. In the case of PHEMA/gelatin IPNs the hydrophilicity of the polymer and macromer chain length as well as the crosslink density of the networks, influence this process.

Swelling studies were performed in the phosphate buffer solution of pH 7.4 at 37^0C, that simulates the physiological conditions. Equilibrium degree of swelling values for simple networks (without gelatin) and IPNs (with gelatin), are plotted as a function of time (Figs. 4 and 5).

Fig. 4. Swelling vs time for gels without gelatin.

Fig. 5. Swelling vs time for gels with gelatin.

Due to their highly porous structure, mass of all cryogel discs increased abruptly after being submerged in phosphate buffer solution for less than 10 minutes. The swollen mass of samples **CG1** and **CG3** increased by 145% and 89%, respectively, and **CG2** by 160%. All cryogels samples without gelatin reached swelling plateau after 4 hours in accordance with their degree of hydrophilicity and their superporous structure, with the equilibrium degree of swelling 2.88 ± 0.04 for **CG1**, 3.38 ± 0.04 for **CG2**, 2.38 ± 0.03 for **CG3** and 2.18 ± 0.03 for **CG4**. Due to the highly hydrophilic nature of **CL2** monomer and lowest crosslinking density, **CG2** absorbed the highest amount of water. As all PBAE macromers were added in the same amount (15% weight of HEMA monomer), **CL2** was added in lowest molar percentage, resulting in lower crosslinking density, which also explains its higher swelling degree. **CL1** has nearly identical chemical structure as **CL2**, but lower molecular weight, resulting in higher crosslinking density, and lower swelling degree of **CG1**, compared to **CG2**. Due to the hydrophobic nature of the hexylene group, **CG3** has the lower swelling rate compared to other PBAE cryogels.

CG4 sample, in which PEGDA was used as a crosslinker, showed lower swelling rate compared to cryogels with PBAE crosslinkers. This can be explained by the positively charged nitrogen in PBAE crosslinker structure and its higher ability to form hydrogen bonds with water molecules, making it more hydrophilic than PEGDA. As it can be seen from Figs 4 and 5, samples with gelatin (**CG5 - CG8**) swell more than those without gelatin, which can be expected due to gelatin's highly hydrophilic nature. Furthermore, as PHEMA/gelatin semi-IPNs are formed during the crosslinking/polymerization of HEMA with PBAE macromers, gelatin molecules move apart PHEMA chains, acting as a pore forming agent. Accordingly, larger pores are formed than in the absence of gelatin, which is also confirmed by SEM micrographs (Fig. 6).

All cryogels with gelatin reached swelling plateau after 5.5 hours, with the equilibrium degree of swelling 4.95 ± 0.10 for **CG5**, 5.92 ± 0.06 for **CG6**, 4.22 ± 0.08 for **CG7** and 3.55 ± 0.07 for **CG8**.

3.5. Cryogel morphology

Morphology of the cryogels was observed by Scanning Electron Microscope. Micrographs showed interconnected polyhedral, elongated pores, with small pores surrounded by polymeric thick walls of macropores. Samples **CG1** and **CG3**, obtained using macromers **CL1** and **CL3**, with a small difference in average molecular weight, show similar layout but with smaller pores for **CG3**, which is probably due to HDDA diacrylate more hydrophobic nature. Samples **CG1** and **CG3** both show small pores interconnected by large canals. **CG2** has largest pores, due to longer chain of **CL2** macromer than in the case of **CG1** and **CG3**. Cryogel with PEGDA, **CG4**, showed the smallest pores due to PEGDA lowest hydrophilicity and lower number-average molecular weight. In general, cryogels without gelatin (Fig. 6. a, b, c, d) have smaller pores compared to their analogous IPN cryogels with gelatin (Fig. 6. e, f, g, h), but both series follow the comparable trend for pore size, which is obviously dictated by the type and nature of the poly(beta amino) ester macromer.

Fig. 6. SEM micrographs for cryogels without gelatin (left): **CG1** (a), **CG2** (b), **CG3** (c), **CG4** (d) and with gelatin (right): **CG5** (e), **CG6** (f), **CG7** (g) and **CG8** (h). (bar – 200 μm).

3.6. Porosity measurements

Convenient pore size is necessary to enable successful cells seeding and diffusion throughout the matrix of both cells and nutrients. Scaffolds must be permeable with interconnecting pores to facilitate cell growth, migration and nutrient flow for cell survival, proliferation, and migration [28]. A relatively high porosity (80–90 %) is also wanted for adequate cell adhesion [29, 30]. The degree of porosity has a great influence on the mechanical properties with the stiffness of the scaffold decreasing as porosity increases [31].

Porosity measurements show an increase of porosity in cryogel samples with gelatin compared to their analogous samples without gelatin. This trend can be explained by the higher swelling ratio of cryogels with gelatin, which have a larger water intake, resulting in larger pore formation from ice crystals during cryogelation. All cryogels containing gelatin show porosity in the range of 80.21-87.04%. Cryogels with **CL2** crosslinker displayed the highest porosity, due to the lowest crosslinking degree (Table 2).

3.7. Mechanical properties

The elastic modulus of the scaffolds is a very important physical property because it determines their use in biomedical applications. Ideally, it should match the mechanical properties of physiological tissue surroundings were the scaffold is implanted and must be strong enough to allow surgical handling during implantation [32].

Mechanical properties of our samples considerably depend on the type of crosslinker, showing similar trends in both sample groups, with and without gelatin (Table 2). Values of Young modulus obtained for hydrogels based on methacrylamide-modified gelatin and poly(ethylene glycol) [3] or methacrylamide-modified gelatin and PHEMA [33, 34], fall in the range of 4.4 to 327.7 kPa and in the case of unmodified gelatin hydrogels even lower values were obtained [35]. In this respect it can be concluded that considerable increase in Young moduli was achieved by producing new gelatin/HEMA IPNs, with moduli values in the range of 3.24 - 4.52 MPa.

Table 2. Equilibrium degree of swelling values, Young's modulus, porosity, percentage of degradation for cryogel samples, and percentage of elongation on break.

Cryogel	Equilibrium degree of swelling	Young's modulus (MPa)	Porosity (%)	Percentage of mass loss	Elongation on break (%)
CG1	2.88 ± 0.04	4.38 ± 0.25	73.85	25.95 ± 1.58	24.42 ± 1.32
CG2	3.38 ± 0.04	3.51 ± 0.21	79.60	32.71 ± 1.65	22.85 ± 1.30
CG3	2.38 ± 0.03	4.21 ± 0.22	72.49	21.4 ± 1.60	27.73 ± 1.43
CG4	2.18 ± 0.03	4.76 ± 0.22	66.93	7.14 ± 0.96	28.24 ± 1.42
CG5	4.95 ± 0.10	4.06 ± 0.20	81.93	20.87 ± 0.94	23.51 ± 1.30
CG6	5.92 ± 0.06	3.24 ± 0.18	87.04	26.54 ± 0.67	20.26 ± 1.04
CG7	4.22 ± 0.08	3.96 ± 0.20	82.79	15.95 ± 0.76	25.69 ± 1.32
CG8	3.55 ± 0.07	4.52 ± 0.24	80.21	4.38 ± 0.81	26.17 ± 1.30

The modulus data are presented in Tab. 2. Generally, IPN samples show slightly lower modulus values, in the range of 3.24 - 4.52 MPa, due to their higher porosity and larger pore size compared to the

samples without gelatin, with modulus value in the range of 3.51 - 4.76. The samples **CG4** and **CG8**, crosslinked with PEGDA, which has the lowest average molecular weight, have highest modulus value, while the samples **CG2** and **CG6** show the lowest modulus values in their respective groups. The values for Young modulus decrease with the increase of average molecular weight of the PBAE crosslinker and are in accordance with the degree of swelling, showing the mutual influence of crosslinker molecular weight and its hydrophilic/hydrophobic nature. Values for elongation at break follow a similar trend to Young's modulus values, in the range of 22.85 - 28.24 % for cryogels without gelatine, with slightly lower values for cryogels with gelatin, in the range of 20.26 - 26.17 %. It can be concluded that mechanical properties of synthesized IPNs can be tuned by changing the crosslinker component.

3.8. Degradation studies

Hydrolytic degradation of polymer networks is generally complex. Main properties that influence the degradation are: crosslink density, hydrophilicity/hydrophobicity, swelling capacity, surface charges and topography, specimen thickness, pH and ionic strength of the solution, and interaction between two hydrogel networks in the case of IPNs. In addition, the relative concentration of the components during polymerization is also important [36].

Another important parameter influencing the hydrolytic degradation of investigated PHEMA/gelatin IPNs and PHEMA networks, is also the type of PBAE macromer. The macromers used in this study were designed in order to obtain tunable rates of degradation, allowing for a variety of degradation profiles for samples with and without gelatin (Fig. 7).

Fig. 7. Percentage of weight loss for cryogels with and without gelatin after 16 weeks.

Among the cryogels with PBAE crosslinkers, **CG3** exhibits the slowest degradation rate, presumably due to the hydrophobic structure of HDDA in **CL3** crosslinker. The more hydrophilic nature of the PBAE crosslinkers with DEGDA, in samples **CG1** and **CG2,** allows more water molecules to enter the cryogel network, resulting in faster degradation. Longer PBAE chains in **CL2** macromer, and lower crosslinking density in **CG2** additionally sped up degradation rates. Therefore, **CG2** scaffold showed the highest rate of degradation, with fractional mass loss of 32.71 ± 1.65 % after the period of 16 weeks. The degradation rates of **CG3** and **CG1** were lower, with respective mass loss of 21.4 ± 1.60 % and 25.95 ± 1.58 % after 16 weeks. **CG4** showed less than 8% degradation (Table 2).

Addition of gelatin slowed down the hydrolytic degradation rates of cryogels. Analogue of **CG2**, cryogel **CG6** showed the highest rate of degradation, with fractional mass loss of 26.54 ± 0.67 % after the period of 16 weeks. The degradation rates of **CG5** and **CG7** had respective mass loss of

20.87 ± 0.94 % and 15.95 ± 0.76 % after 16 weeks. In the same period sample **CG8** showed less than 5% degradation.

3.9. Biocompatibility testing

Results of biocompatibility testing showed a very satisfying biocompatibility of the tested hydrogels containing gelatin (Fig. 8). Biocompatibility is expressed as a high percent of viable normal human fibroblast after 24 hours treatment with prepared cryogel supernatants. Viability of MRC5 cells after treatment with prepared cryogels-supernatants was higher than 90 % for all test samples with gelatin, showing 20% or higher increase in viability, compared to hydrogels without gelatin.

Fig. 8. Effect of supernatants of cultivated cryogels to viability of normal human fibroblast (MRC5 cell line).

Results represent mean +/- standard error of percent of viable cells after 24h treatment with supernatants of new synthetized hydrogels. This is representative experiment of two/three performed experiments.

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4. Conclusion

Novel degradable HEMA/gelatin based IPNs with different PBAE crosslinkers were successfully fabricated and characterized. Additional cryogel samples without gelatin were synthesized to assess the effects of gelatin addition and IPN formation on cryogel properties. Cryogels displayed different morphologies, porosity, and swelling and degradation rates based solely on the structure of PBAE crosslinker. The addition of gelatin increased the porosity of the cryogels, along with the swelling and degradation rates, which resulted in slightly reduced mechanical strength. All cryogels containing gelatin showed great biocompatibility, with MRC5 viability (over 90%).

The results obtained indicate that PHEMA/gelatin IPNs have favourable swelling and mechanical characteristics, along with slow degradation rates, which can be tuned by changing PBAE crosslinker composition or molecular weight, for the potential application as scaffolds in tissue

regeneration. Aditional studies are required to assess biocompatibility and degradation of the cryogel samples under *in vivo* conditions.

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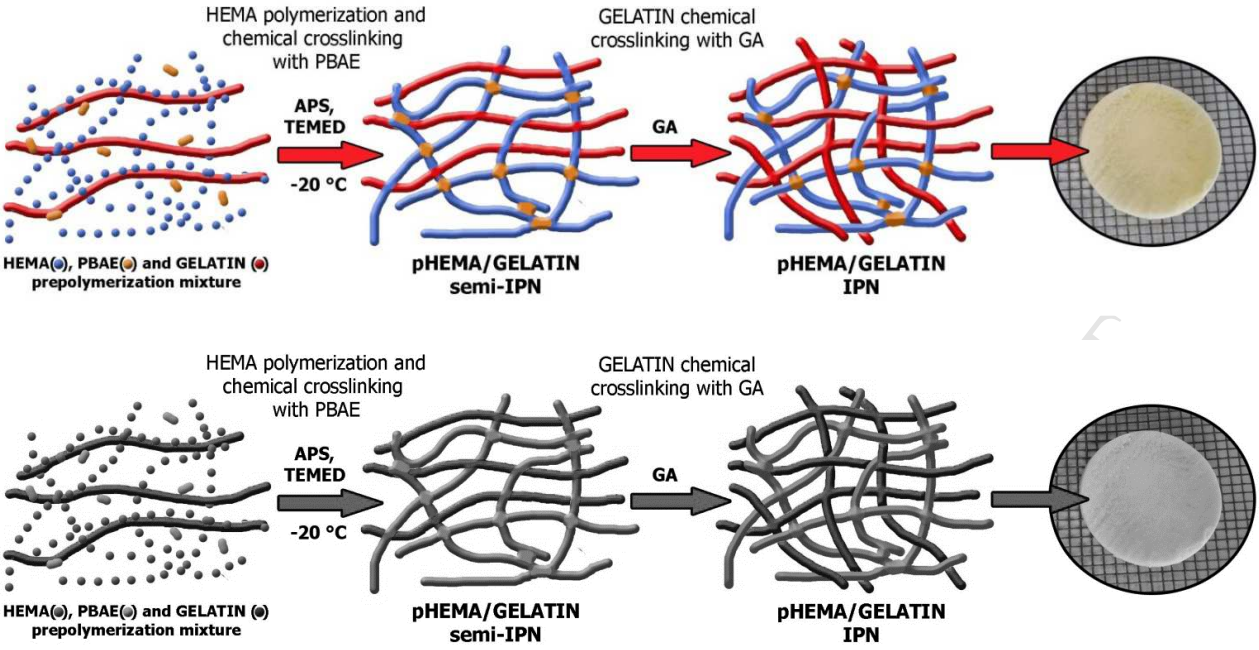

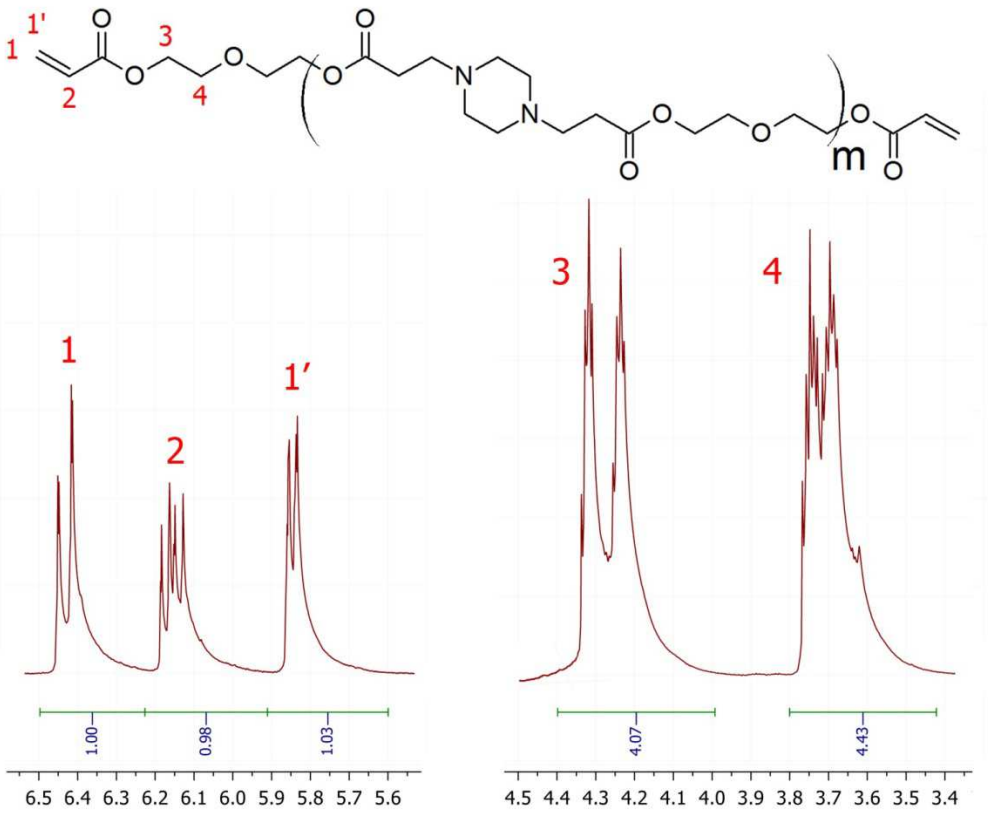
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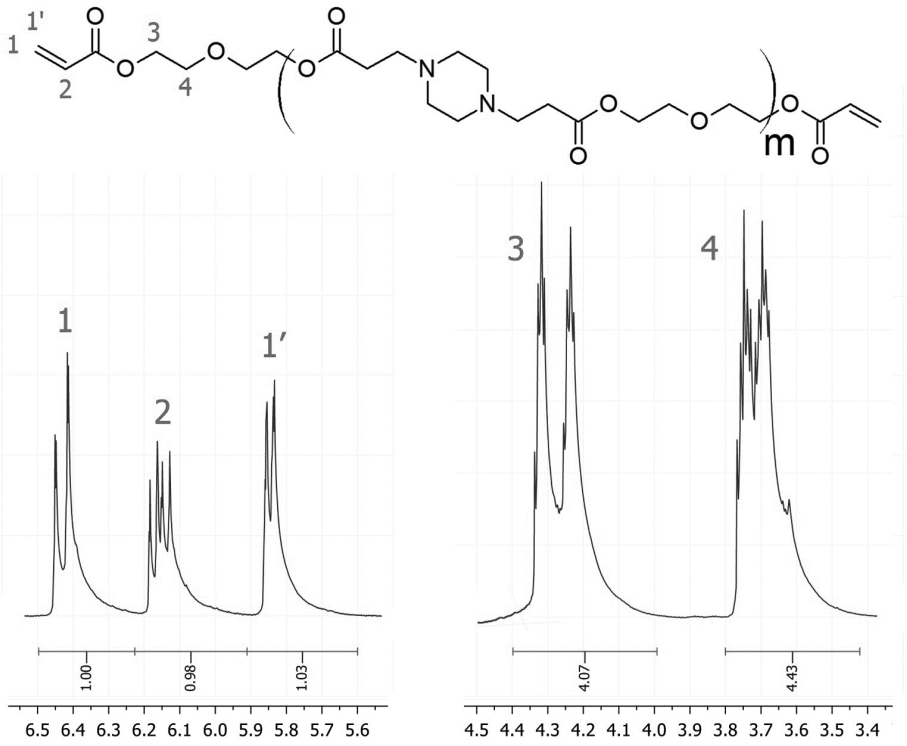
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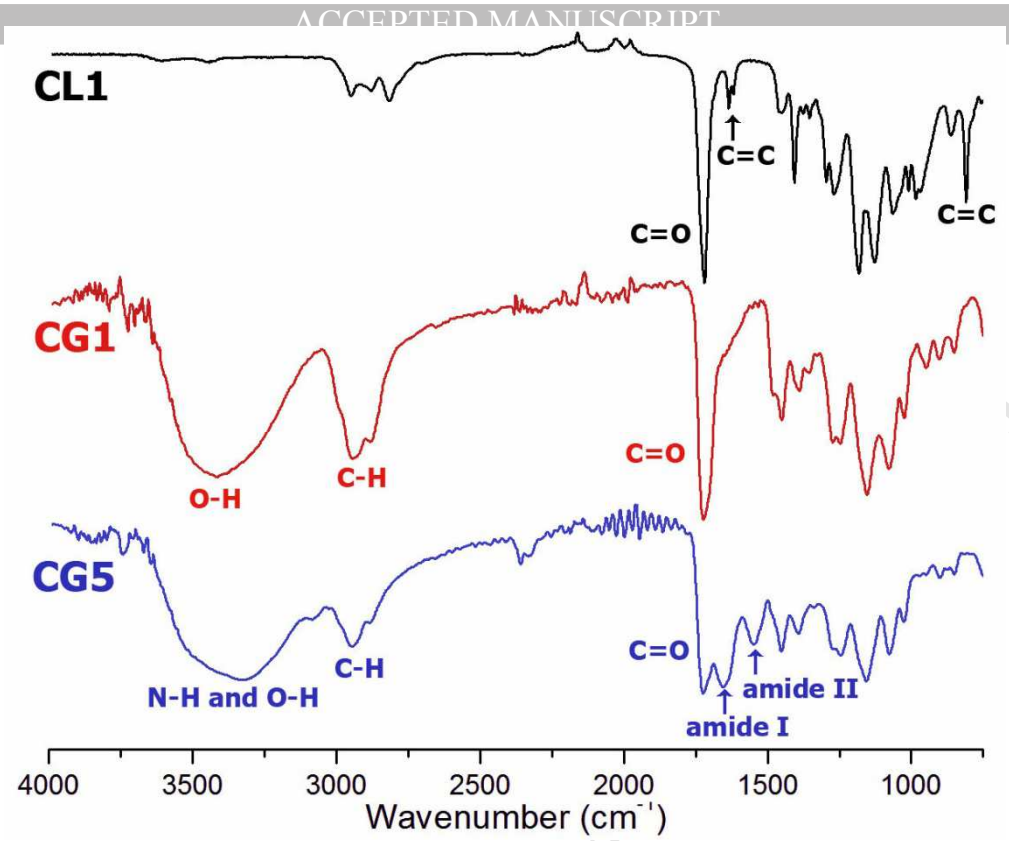

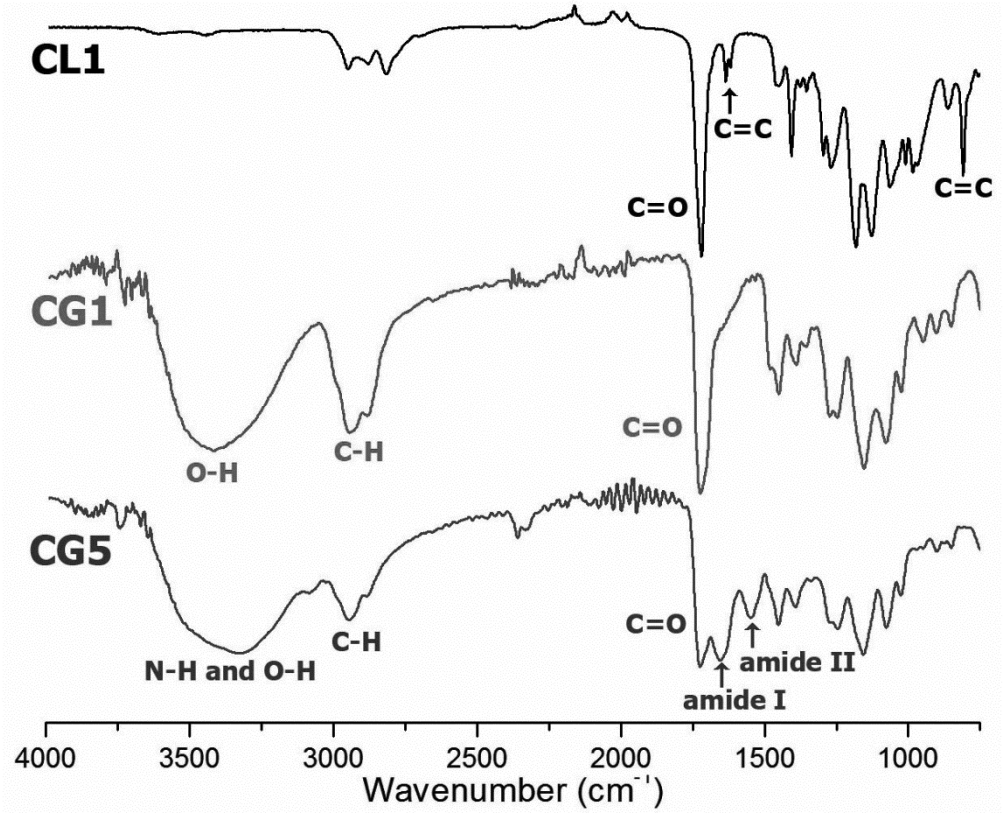

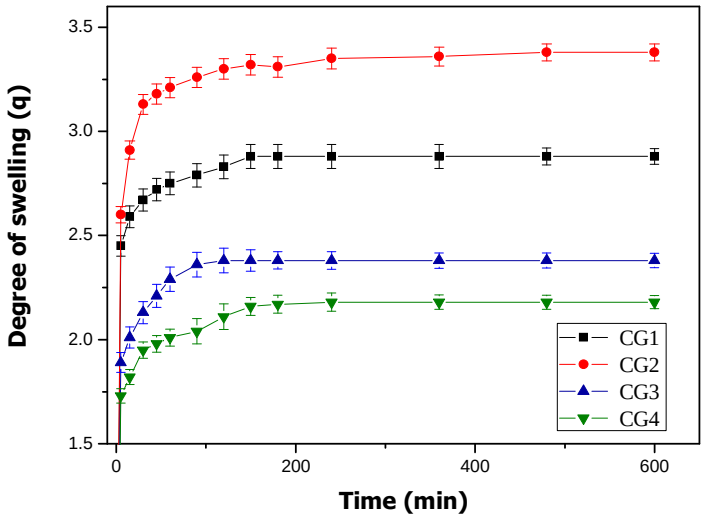

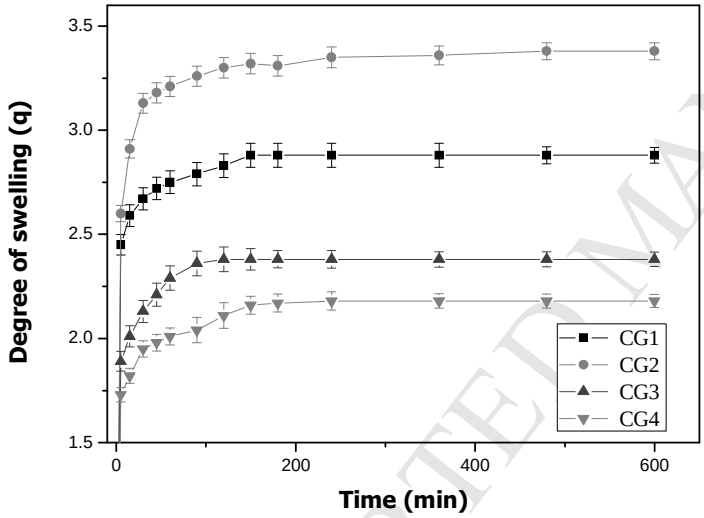

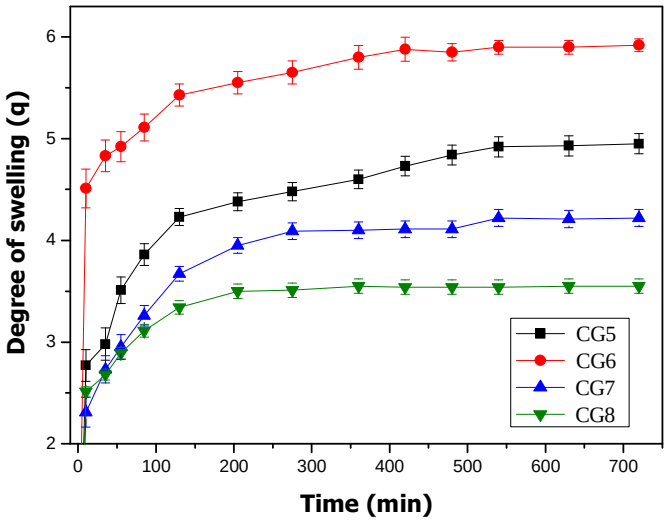

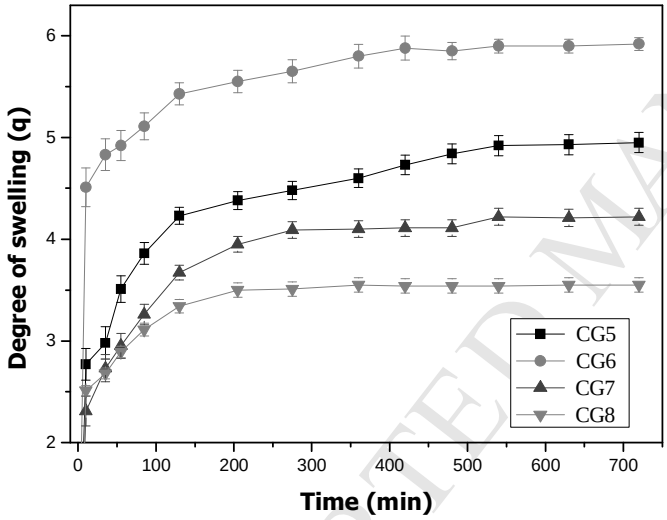

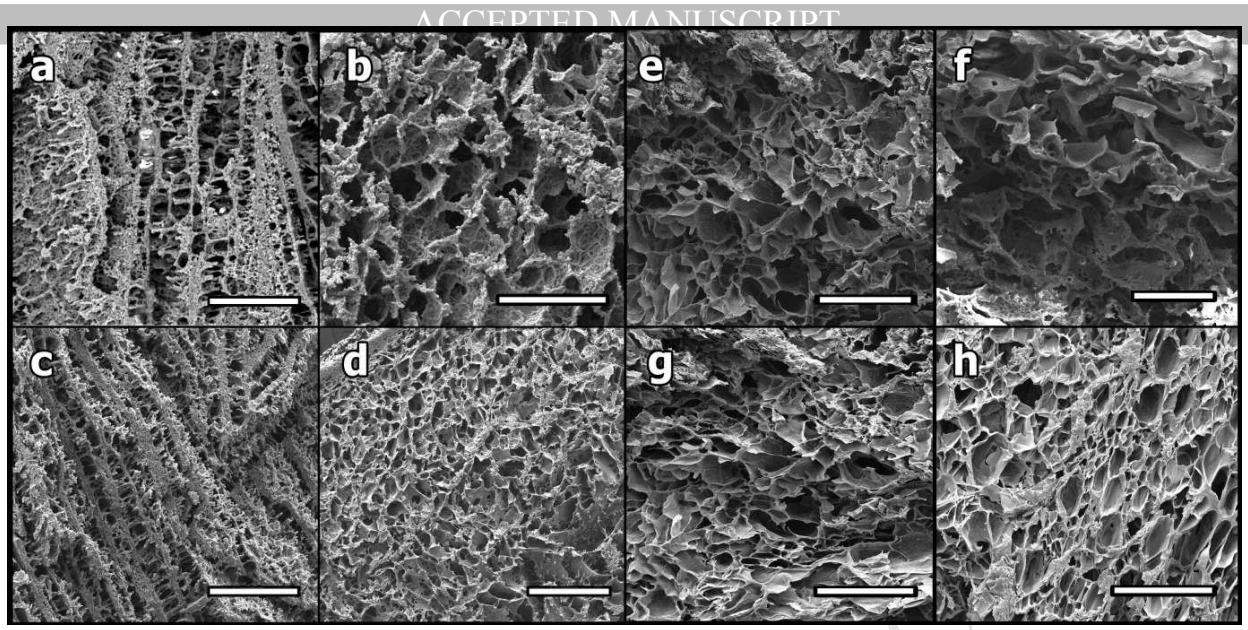

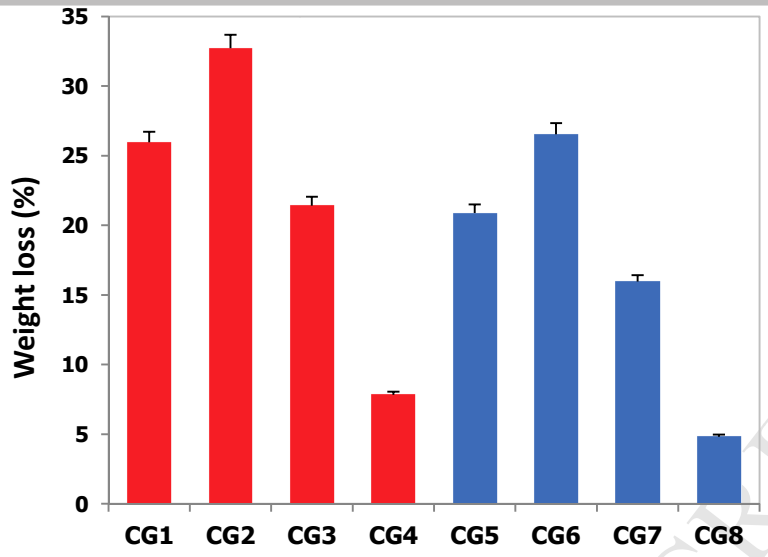

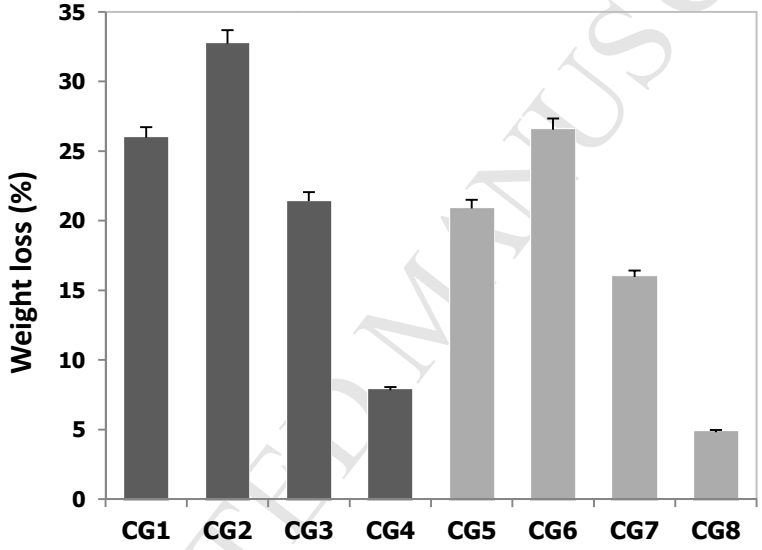

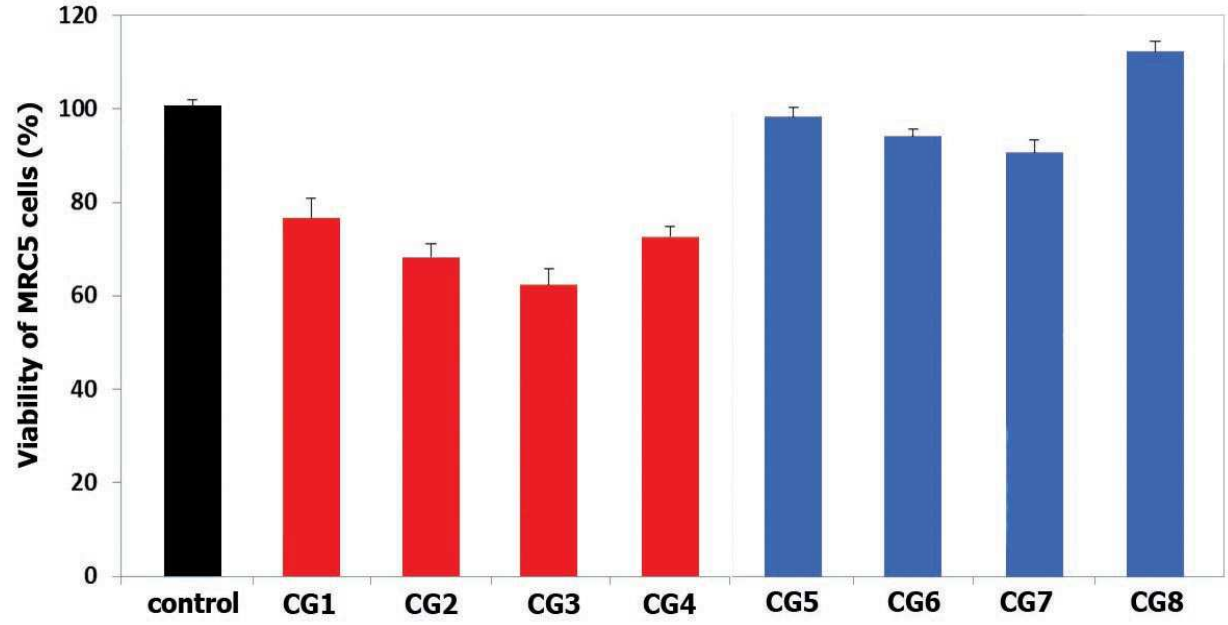
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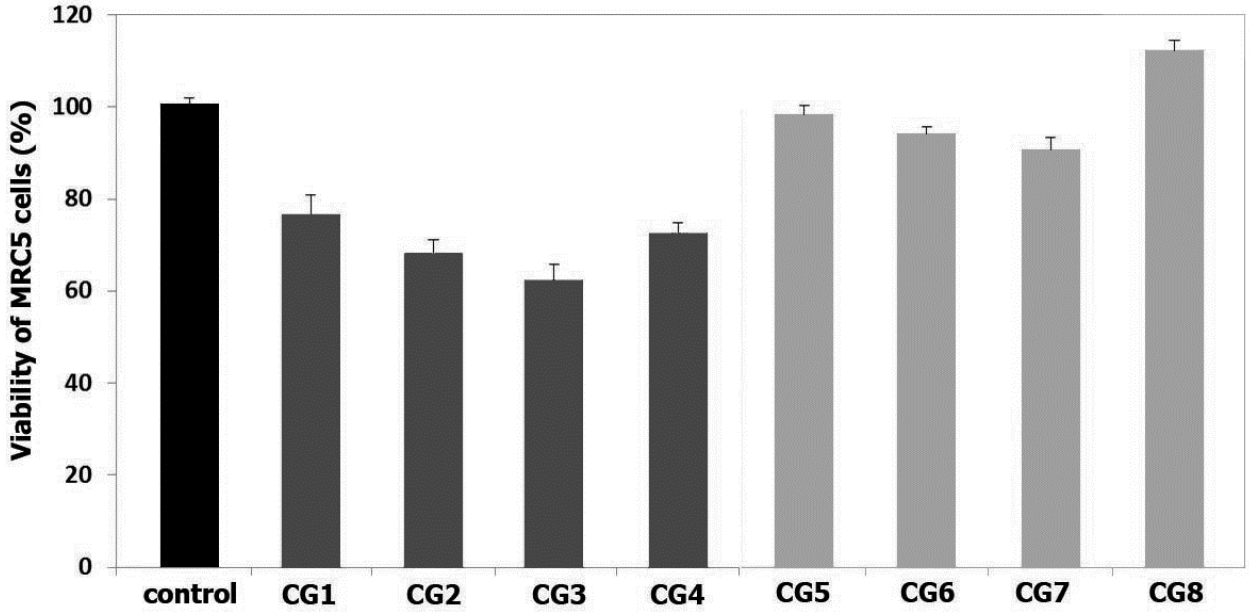
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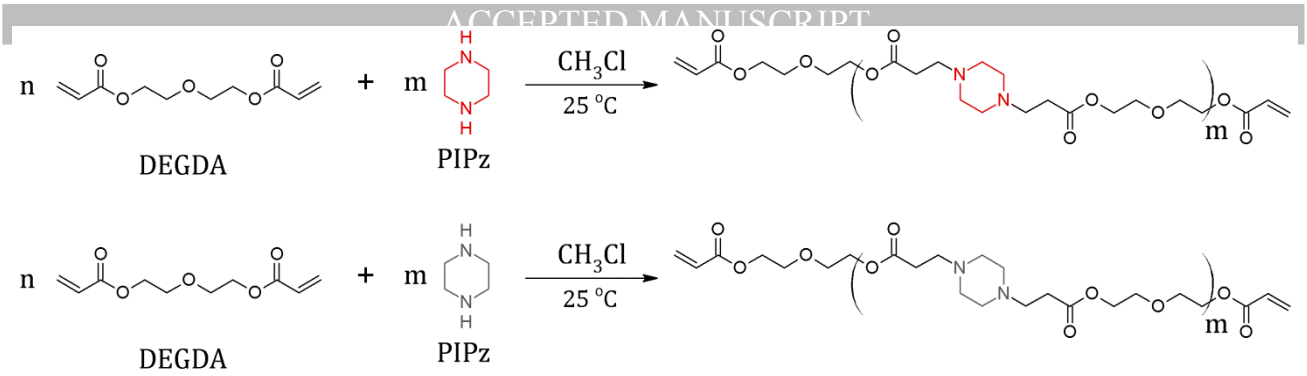

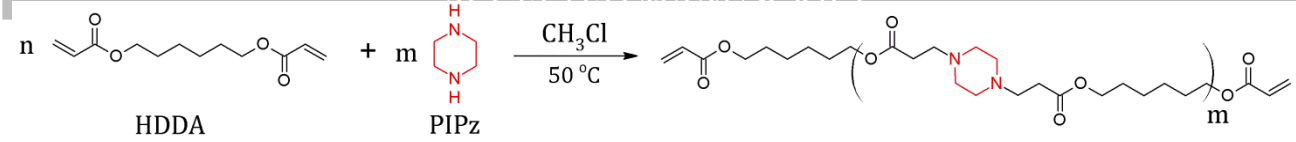

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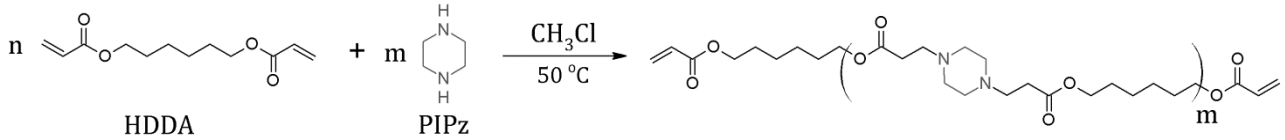

HDDA PIPz

Synthesis and characterization of novel biodegradable poly(2-hydroxyethyl metacrylate)/gelatine based IPN scaffolds.

Tunable IPN scaffold properties with different PBAE crosslinkers for HEMA networks.

The advantages of gelatine containing IPNs, due to the combination of natural and synthetic hydrogel, confirmed by comparison with a second set of cryogels without gelatine.

High viability with MRC5 cells validated for all novel IPNs.