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Preparation of collagen modified photopolymers: a new type of biodegradable gel for cell growth

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Abstract In this study a new branched methacrylated poly(propylene glycol-co-lactic acid) (PPG–PLA–IEM) and methacrylated cellulose acetate butyrate resin (CAB–IEM) were synthesized. Hydrogels with various amounts of PPG–PLA–IEM and CAB–IEM (25, 50 and 75 wt% IEM modified) were prepared by photopolymerization. Collagen tethered PEG–monoacrylate (PEGMA–collagen) was prepared and introduced as a bioactive moiety to modify the hydrogel in order to enhance cell affinity. In vitro attachment and growth of 3T3 mouse fibroblasts and human umbilical vein endothelial cells (HUVEC) on the hydrogels with and without collagen were also investigated. It was observed that, the collagen improves the cell adhesion onto the hydrogel surface. With the increasing amount of collagen, cell viability increased by 28% for ECV304 ($P < 0.05$) and 30% for 3T3 ($P < 0.05$).

1 Introduction

Tissue engineering deals with the development of appropriate organs or tissues to replace, support or heal the parts of the body which have been damaged and are not functioning properly [1, 2]. Every year millions of people suffer from bone and soft tissue lesions [3]. The use of autograft (grafted sections of tissue harvested from the body of the patient), allograft (grafted tissue is taken from cadavers) or synthetic materials are the most common treatments for reconstruction of the damaged tissue [4, 5]. All the aforementioned treatments have limitations due to the risk of donor-site morbidity, potential rejection of foreign tissue due to mismatch of mechanical properties, and the possible transmission of disease [6]. Because of these limitations current research to has been focused on the use of scaffolds that can provide a temporary platform for cell growth and can be degraded over a time as new tissue develops [7]. Scaffolds can be seen as an artificial extracellular matrix and polymers constitute a major class of materials for developing scaffolds in tissue engineering applications. Polymeric scaffold material must fulfill a number of requirements including biocompability, biodegradability to non toxic by-products, controllable degradation rate, processability to desired shapes, appropriate porosity and mechanical properties, and where desirable, the promotion of cellular attachment, colonisation, and proliferation and proliferation [8, 9]. While biodegradable scaffolds offer the possibility to create completely natural new tissue and replace new organ function, scaffolds are mainly chosen from biodegradable polymeric materials [10]. Among the biodegradable polymers, aliphatic polyesters such as poly(α-hydroxy acids), poly(lactic acids) and biopolymer based materials such as cellulose, starch, proteins, to lignin, and polysaccharides are the leading class of degradable polymers [11, 12]. Functionalization of these polymers with desired end groups is necessary to acquire the required performance to prepare the scaffold. Many techniques have been used to produce scaffolds such as solvent casting,

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phase inversion/inversion, fiber bonding, melt-based technologies, high pressure-based methods, freeze drying, electro spinning, and UV curing [13–19]. Among these methods, photo-initiated crosslinking was found to be the most rapid and effective way for solidification of injectable formulations [20]. Injectable, biodegradable scaffolds based on poly(1,2-propylene fumarate), polyethylene glycol (PEG) acrylates/methacrylates, methacrylated polyanhydrides and modified polysaccharides which contains crosslinkable sites in their backbone are suitable materials for photo-crosslinking and have attained much interest [21–24]. They are also the most suitable materials due to their ability to expand within the defected area and fill the whole cavity.

A number of reports have been published on methacrylated PEG, PPG, PLA and dextran [22–25]. But within the literature, there are no studies about urethane–cellulose acetate butyrate polymers. Cellulose acetate butyrate (CAB) is one of the most important thermoplastic cellulose esters, and it can be biodegraded in environment [26]. Introduction of methacrylic double bonds into the CAB make it crosslinkable through photopolymerization. Studies about dimethacrylates have shown that crosslinking by methacrylic groups are biocompatible. Besides that unreacted dimethacrylates have relatively low cytotoxicity [22–27]. Poly(D,K-lactic acid) have free hydroxyl groups which can be converted to methacrylic groups like CAB. Most of the studies for the introduction of double bonds to PLA are focused on acrylation with acryloyl chloride [28]. 2-isocyanatoethyl methacrylate (IEM) is a methacrylation agent that incorporates urethane linkages and methacrylic groups onto polymer backbones. Among the studies about tissue engineering applications IEM was only used to modify PEG. Besides the methacrylic groups, urethane linkages are also providing an additional adjustable parameter controlling polymer properties [22].

In this study, a star type methacrylated poly(propylene glycol-co-lactic acid) (PPG–PLA–IEM), and methacrylated cellulose acetate butyrate resin (CAB–IEM) were synthesized. Hydrogels with various amounts of PPG–PLA–IEM and CAB–IEM (25, 50 and 75 wt% methacrylic modified) were prepared by photopolymerization. In addition, a collagen tethered PEG–monoacrylate (PEG-MA–collagen) was synthesized and it was introduced as a bioactive moiety to modify the hydrogel structure in order to enhance cell affinity. 3T3 mouse fibroblast cells and human umbilical vein endothelial cells (ECV304) were cultured on the hydrogels. Viability of the cells was also determined using a quantitative MTT assay in order to evaluate the ability of hydrogels to support cell growth and proliferation.

2 Materials and methods

2.1 Materials

Poly(propyleneglycol) diglycidyl ether, [PPGDGE] (M_n = 380); poly(ethylene glycol) diacrylate, [PEGDA] (M_n = 258), 2-isocyanatoethyl methacrylate [IEM] and 3,6-dimethyl-1,4-dioxane-2,5-dione [D,L-lactide] were purchased from Aldrich Chemical Co. Cellulose acetate butyrate [CAB-381-20 Eastman] was kindly provided by a local user. Collagen (TypeI), 1,1′-carbonyldiimidazole [CDI], triphenyl phosphine, [TPP], *N*-vinyl pyrrolidone [NVP] and 2,2′-bis(hydroxymethyl) propionic acid [DMPA] was purchased from Fluka AG. The photoinitiator, 1-hydroxy cyclohexyl phenyl ketone [Irgacure-184], was kindly provided by Ciba Speciality Chemicals, Turkey. Dibutyltin dilaurate and tin(II) 2-ethylhexanoate [Sn(II)Oct] were kindly supplied by Henkel, Turkey. [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma. All other chemicals were of analytical grade and were purchased from Merck AG. Freshly double distilled water was used throughout the experimental work.

2.2 Preparation of prepolymer (PPG–DMPA)

Poly(propylene glycol) diglycidyl ether (10 g) and triphenyl phosphine (0.05 g) were put into a three-necked 100 ml of round-bottom flask equipped with a mechanical stirrer, a condenser and a nitrogen inlet. The reaction vessel was placed in an oil bath and was heated to 50°C while stirring. Then, 2,2′-bis(hydroxymethyl) propionic acid was added to PPGDGE slowly. After the addition was completed, the reaction mixture was kept at 90°C for 6 h. The product was a clear viscous liquid.

2.3 Synthesis of poly(propylene glycol-co-lactic acid) copolymer (PPG–PDLA)

Synthesis of poly(propylene glycol-co-lactic acid) (PPG–PDLA) polymer was performed by a ring-opening polymerization method. PPG–DMPA (0.5 g) and freshly recrystallized D,L-lactide (5 g) were reacted at 110°C for 24 h in a vacuum sealed glass tube using tin (II) 2-ethylhexanoate (Sn(II)Oct) as a catalyst (monomer to catalyst, mole ratio, M/C = 1000). The glass tube was cooled to room temperature then it was opened. The reaction mixture was purified by dissolving in a small amount of dry dichloromethane and dropping into an excess of hexane. Precipitation is effected by addition of 0.05 g calcium

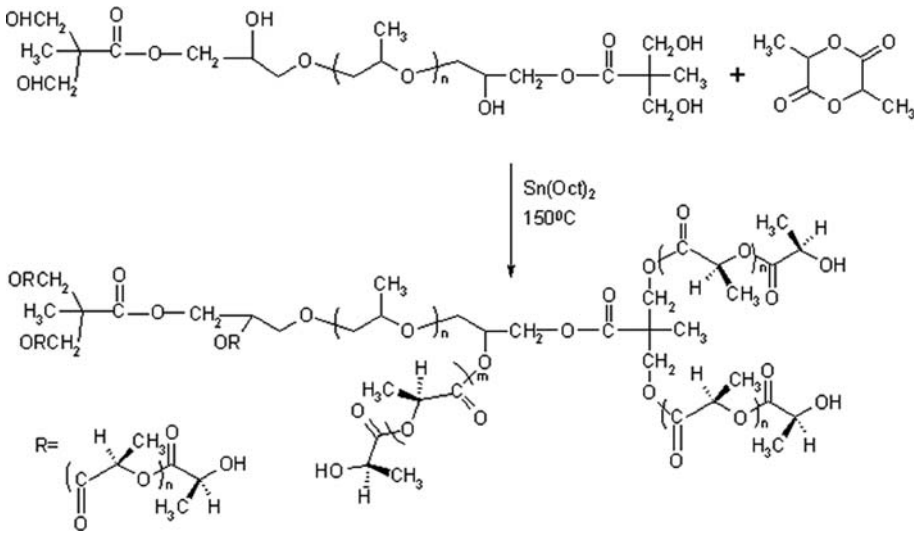
chloride. This precipitate is collected by filtration and drying under vacuum (0.5 mbar) at 10°C. The conversion of polymer was 70% and the average molecular weight (M_n) of the PPG–PDLA copolymer was found to be 17000 g mol^{-1} using gel permeation chromatography (GPC). The synthesis of poly(propylene glycol-co-lactic acid) copolymer was illustrated in Scheme 1.

2.4 Methacrylation of poly(propylene glycol-co-lactic acid) copolymer (PPG–PLA–IEM)

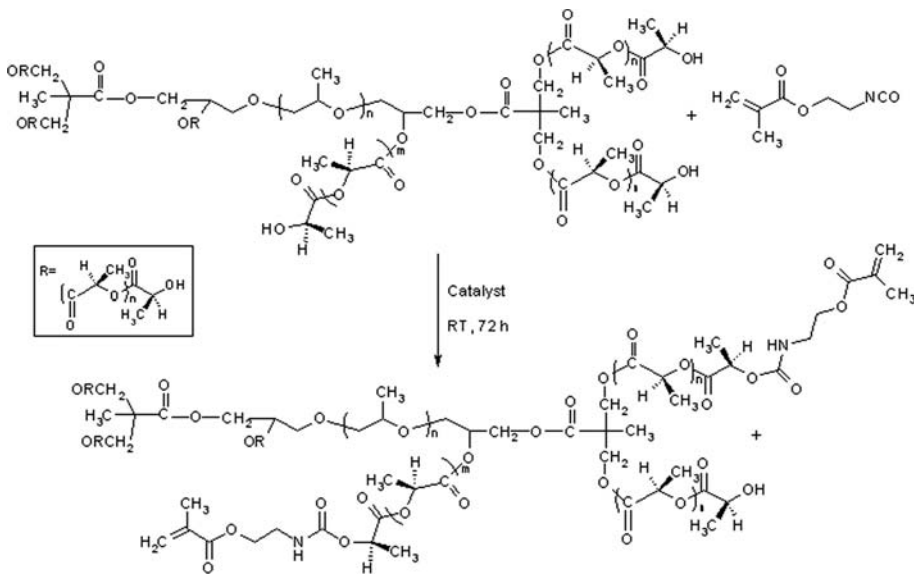
PPG–PDLA (10 g) and 50 ml dry dichloromethane (CH_2Cl_2) were poured into a 250 ml of flame dried three-necked round-bottom flask, equipped with a nitrogen inlet and a dropping funnel. After PPG–PDLA was homogeneously dissolved in CH_2Cl_2, 0.4 g 2-isocyanatoethyl methacrylate (IEM), equivalent to 50 wt% of theoretical hydroxyl content of PPG–PDLA, was added drop wise into the well-stirred reaction mixture. The reaction mixture was kept at room temperature for 72 h. Disappearance of the characteristic –NCO peak at 2275 cm^{-1} in the FT-IR spectrum confirmed that the reaction was completed. The polymer was precipitated in hexane, filtered and dried under vacuum (0.5 mbar) at 10°C for 4 h. The product then kept at −18C°. Synthesis of methacrylated of star type poly(propylene glycol-co-lactic acid) copolymer was illustrated in Scheme 2.

2.5 Methacrylated cellulose acetate butyrate resin (CAB–IEM)

CAB–IEM was prepared through reacting CAB-381-20 and IEM with various feeding ratios as given in Table 1. The preparation of CAB–IEM-50 is given as an example.



Scheme 1 Schematic representation of synthesis of poly(propylene glycol-co-lactic acid) copolymer



Scheme 2 Schematic representation of synthesis of methacrylated of star type poly(propylene glycol-co-lactic acid) copolymer

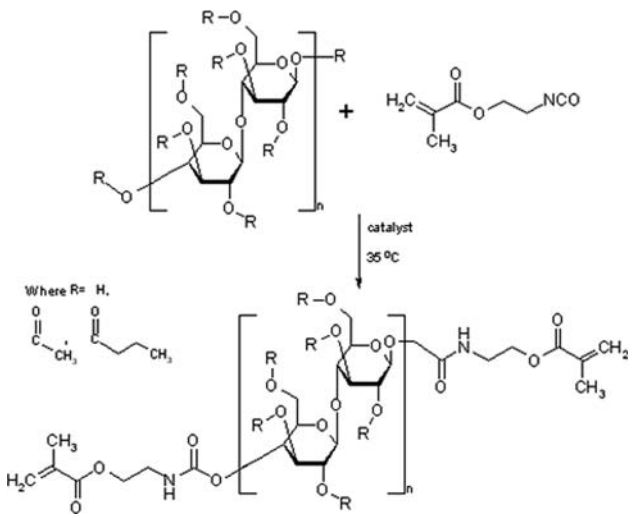
Table 1 The modification ratio of CAB-381-20

Sample	CAB-381-20 (g)	IEM (g)	DBTDL (wt%)
CAB–IEM-25	10	0.185	0.1
CAB–IEM-50	10	0.370	0.1
CAB–IEM-75	10	0.555	0.1

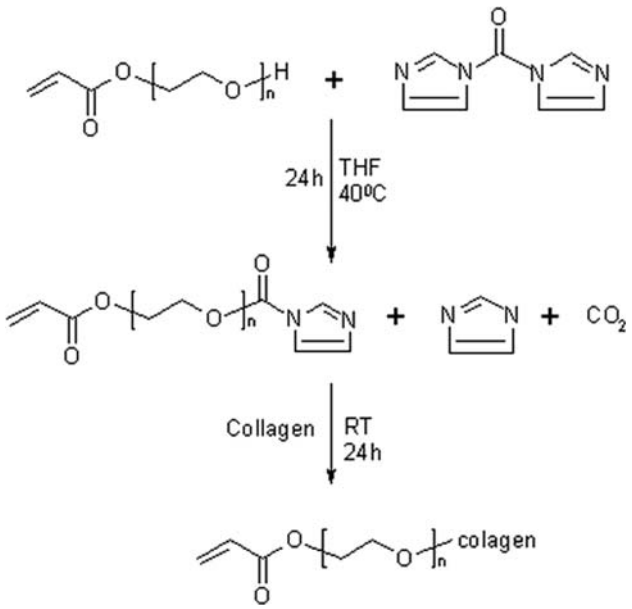
CAB-381-20 (10 g) was dissolved in 100 ml of dry 2-butanone (MEK) homogenously and then the solution was poured into a 250 ml of flame dried three-necked, round-bottom flask, equipped with a nitrogen inlet and dropping funnel. Dibutyltin dilaurate (DBTDL) (0.1 wt%) was added into the reaction flask as a catalyst and the system was heated to 40°C. Separately, IEM (0.37 g), equivalent to 50 wt% of theoretical hydroxyl content of CAB-381-20 was dissolved in 10 ml of dry MEK and then added drop wise to the well-stirred reaction mixture. The reaction mixture was kept at 40C° for 4 h. Disappearance of the characteristic –NCO peak at 2275 cm^{-1} in the FT-IR spectrum confirmed that the reaction was completed. The product was precipitated in excess methanol, filtered and dried under vacuum (0.5 mbar) at 35°C. Synthesis of methacrylated cellulose acetate butyrate resin was shown in Scheme 3.

2.6 Preparation of collagen tethered PEGMA

Polyethylene glycol mono acrylate (PEGMA, 11 g) was poured into a three-necked 100 ml of round-bottom flask equipped with a magnetic stirrer, a condenser and a nitrogen inlet. 1,1-carbonyldiimidazole (CDI, 5.5 g) was dissolved in 50 ml of THF then added drop wise into the reaction mixture while mixing. The reaction mixture was then kept at 40°C for 24 h.



Scheme 3 Schematic representation of synthesis of methacrylated cellulose acetate butyrate resin



Scheme 4 Schematic representation of attachment of collagen into PEGMA

PEGMA–CDI was (1 g) charged into a 25 ml of round-bottom flask equipped with a magnetic stirrer. Collagen solution with a concentration of 2 mg ml^{-1} H_2O was added to the PEGMA–CDI. The reaction mixture was stirred at room temperature for 24 h. Then the product was transferred into a dark bottle and kept at +4°C. The collagen tethering to the PEGMA was illustrated in Scheme 4.

2.7 Characterization of the resins

The structure of copolymers and collagen tethered PEGMA was characterized by FT-IR spectrophotometry. FT-IR spectra were recorded on a Shimadzu 8300 FT-IR spectrophotometer. Samples were dissolved in THF, applied onto KBr discs then the THF was evaporated. 20 scans were performed for each sample. The average molecular weight and molecular weight distribution of the PPG–PDLA copolymer were determined by gel permeation chromatography (GPC) using a set up consisting of the Agilent pump and refractive-index detector (Model 1100) and four Waters Styragel Columns (HR 5E, HR 4E, HR3, and HR2). THF was used as the eluent at a flow rate of 1 ml min^{-1} at 25°C. The molecular weight (MW) of the polymer was calculated with the aid of PSt standards [29].

2.8 Synthesis and characterization of hydrogels

Methacrylated poly(propylene glycol-co-lactic acid) (PDLA–CAB–IEM), methacrylated cellulose acetate butyrate resin (CAB–IEM), *N*-vinyl pyrrolidone (NVP), poly (ethylene glycol) diacrylate (PEGDA), collagen tethered polyethylene

Table 2 The composition of the hydrogel formulations

	PPDMPA–PDLLA–IEM (g)	NVP (g)	CAB–IEM (g)			PEGDA (g)	PEGMA–collagen (g)	Photoinitiator (g)	H_2O (g)
			25	50	75				
PDLA–CAB–IEM-25-0	1	3	0.5			0.45	0	0.135	0.72
PDLA–CAB–IEM-25-1	1	3	0.5			0.45	0.18	0.135	0.72
PDLA–CAB–IEM-25-2	1	3	0.5			0.45	0.36	0.135	0.72
PDLA–CAB–IEM-50-0	1	3		0.5		0.45	0	0.135	0.72
PDLA–CAB–IEM-50-1	1	3		0.5		0.45	0.18	0.135	0.72
PDLA–CAB–IEM-50-2	1	3		0.5		0.45	0.36	0.135	0.72
PDLA–CAB–IEM-75-0	1	3			0.5	0.45	0	0.135	0.72
PDLA–CAB–IEM-75-1	1	3			0.5	0.45	0.18	0.135	0.72
PDLA–CAB–IEM-75-2	1	3			0.5	0.45	0.36	0.135	0.72

glycol mono acrylate (PEGMA–collagen) and photoinitiator (Irgacure184) containing hydrogel formulations were prepared as given in Table 2.

Formulations were poured into Teflon® moulds (diameter 5 mm, height 3 mm) and cured under high pressure UV lamp (OSRAM 300 W, $\lambda_{max} = 365$ nm) for 3 min, then the polymeric discs were removed from the moulds.

For purification, the polymeric discs were dipped into a large excess of distilled water for 24 h to remove unreacted monomers or other residuals then dried to a constant weight in a vacuum oven (150 mbar) at room temperature. The dry discs were weighed (W_i) and immersed in distilled water at room temperature (20.0 ± 0.1°C). After 48 h, the swollen gels were removed from water, gently dried using tissue paper and weighed (W_f). All the samples were lyophilized at −80°C and weighed (W_d).

The degree of gelation was calculated by using the formula:

$$\text{Gelation } (\%) = (W_d/W_i) \times 100 \quad (1)$$

The degree of swelling was calculated by using the formula:

$$\text{Swelling } (\%) = [(W_f - W_d)/W_d] \times 100 \quad (2)$$

2.9 Degradation studies of hydrogels

The in vitro degradation studies of the hydrogels were conducted at 37°C in phosphate buffer saline (PBS, pH 7.4). The vacuum dried gels were replaced in vials containing 15 ml PBS then incubated at 37°C during 6 months. The buffer solution was replaced every 72 h. After incubation the gel was taken out the vial and dried using tissue paper then weighed. The degradation rate was determined by the ratio of the weight loss to the initial weight of the hydrogel.

$$S = (W_i - W_f/W_i) \times 100 \quad (3)$$

where S indicate the rate of degradation, W_i and W_f indicates initial and final weight of the hydrogel, respectively.

2.10 Differential photocalorimetry

Polymerization of hydrogel formulation was carried out by Pyris Diamond DSC equipped with EXFO Omni-Cure™ 2000 photo-DSC accessory. Filtered light (250–450 nm) with an intensity of 20 mW/cm^2 at the tip of the light guide was used. Approximately 15 mg of the sample was placed in the aluminum DSC pans. The polymerization was carried out under a nitrogen flow of 20 ml min^{-1} at 37°C. The time to reach the maximum polymerization heat (t_{max}), the double bond conversion (DBC) and the maximum rate of polymerization (Rp) were determined.

2.11 Rheometry

The viscosities of the uncured formulations were measured using Brookfield RV DV-II + Pro Viscometer. The formulation was placed into the mould (20 mm diameter and 70 mm depth) positioned on the temperature-controlled plate. The temperature was set to 37°C. The viscosity was measured with the spindle speed of 20 rpm.

2.12 Contact angle measurements

Static water contact angle was measured by sessile drop analysis (DSA 10, KRüSS GmbH) to investigate the wettability of the film samples. During the determination of the sessile contact angles, a drop of water (10 µl) was placed by a 10 ml microsyringe on the surface of the dry polymeric discs and its image was captured by a video camera. For each sample 10 measurement were taken. The contact angle was measured as the angle between the interfaces of polymeric disc–water–air.

2.13 Cell culture

Two kinds of cell culture studies were performed using two different types of cells. In first study, 3T3 mouse fibroblasts

and in the second study Human Umbilical Vein Endothelial Cells (HUVEC-ECV304) were used. CEV304 and 3T3 cell lines were obtained from the American type Culture Collection (ATCC). The cells were cultured in DMEM (Dulbecco's modified Eagle's medium-Sigma) supplemented with 10% fetal bovine serum (Sigma), penicillin (50 U ml^{-1})/streptomycin (50 μg ml^{-1}) at 37°C in a 5% CO_2 atmosphere. Once 80% of confluency was reached, cultures were incubated in 0.25% trypsin (Gibco) for 30 min at 37°C, harvested and were diluted to 10×10^5 cells ml^{-1}.

For cell seeding, hydrogels were prepared as described in Sect. 2.8. The hydrogels were sterilized using 70% ethanol for 30 min and placed in 6-well tissue culture plates. Culture plates containing the samples were washed with sterile PBS buffer (pH 7.4) at room temperature to remove the alcohol using a washing bottle. Cells were seeded on the surface of the hydrogels at an approximate density of 10^5 cells ml^{-1}. To investigate the effect of time on cell attachment, cell seeded hydrogels were kept in cell culture for 24, 48 and 72 h.

After one, two and three days of culture, cell seeded hydrogels were taken out from the medium, rinsed twice with sterilized PBS solution and the cells were fixed with 2.5% glutaraldehyde for 20 min. After fixation, the hydrogels were washed with PBS solution again. Hydrogels were placed into a vacuum chamber where the lyophilization process takes place. Water is removed by rapid cooling under vacuum (0.04 mbar) at −80°C for 20 min. Scanning electron microscopy (SEM) was used to investigate cell-hydrogel interaction using a SEM JOEL JSM-5910 LV at 20 kV and 1.2 cm working distance. The fixed specimens were lyophilized at −80°C. After drying, the specimens were coated with gold approximately 300 Å using an Edwards S 150 B sputter coater and observed under SEM.

2.14 Cytotoxicity

For the cytotoxicity test, polymeric discs were sterilized by 70% ethanol, rinsed with PBS (Phosphate Buffer Saline pH 7.4) and DMEM and then placed into 24-well microplate as triplicate. The cytotoxicity assay performed using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as described elsewhere [30]. On the third day of culturing, 5 mg ml^{-1} MTT in serum-free medium solution was added to each well containing the cell-hydrogel construct. Active mitochondria metabolize the tetrazolium salt to form an insoluble formazin dye. After 4 h of incubation, 0.04 N HCl in isopropanol was added. The constructs were broken apart using a tissue homogenizer [31] and absorbance was read with a ELISA spectrophotometer (Kayto) at 560 nm overnight. All experiments were performed in triplicate. % Viability was determined by using the formula:

$$\text{Viability}\,(\%) = (\text{Mean Absorbance of Sample}/\text{Mean Absorbance of Control}) \times 100 \quad (4)$$

2.15 Scanning electron microscopy

Cell-hydrogel interactions were investigated with scanning electron microscopy (SEM). Cell cultured polymeric discs were examined on SEM JOEL JSM-5910 LV.

2.16 Statistical analysis

Cell viability data were analyzed using unpaired *t*-test and presented as mean ± standard deviation (SD) for number of three replicates ($n = 3$). Analysis of variance (ANOVA) was used to access statistical significance and comparison of the data. A P-value < 0.05 was considered significant.

3 Results and discussion

Poly(propylene glycol) diglycidyl ether was reacted with 2,2′-bis(hydroxymethyl) propionic acid in the presence of triphenylphosphine and the structure of obtained resin was investigated by FT-IR spectrum (Fig. 1).

As seen in Fig. 1, the appearance of the characteristic ester carbonyl band at 1730 cm^{-1} and a broad band about 3400 cm^{-1} which is assigned to secondary-hydroxyl (−OH) groups indicates the opening of the epoxy rings. Besides that, the disappearance of the characteristic epoxy band at 835 cm^{-1} indicates the 100% opening of the epoxy rings.

FT-IR spectrum of the PPG–PDLA copolymer is shown in Fig. 2. As can be seen in Fig. 2, the broad band at 3400 cm^{-1} had disappeared due to reaction of hydroxyl groups with D,L-lactide.

Then the PPG–PDLA copolymer was reacted with isocyanato ethyl methacrylate (IEM) in the presence of dibutyl tin dilaurate as a catalyst. As seen in Fig. 3, the complete disappearance of the absorption band at 2275 cm^{-1}, which is assigned to the characteristic isocyanate group, confirmsthe completion of the reaction between IEM and PPG–PDLA copolymer. It also shows the characteristic =N–H stretching band at 3335 cm^{-1} and C=C double bond vibrations of methacrylate group at 1635 and 810 cm^{-1}.

CDI is a well known coupling agent for amino acids or peptides to hydroxyl terminated materials such as poly(ethylene glycol). In this study *N*-acylimide terminated PEGMA was synthesized via the reaction between CDI and the mono functional PEGMA. The FT-IR spectrum of the *N*-acylimide terminated PEGMA is given in Fig. 4. While the –OH stretching band disappeared at 3400 cm^{-1}, a new

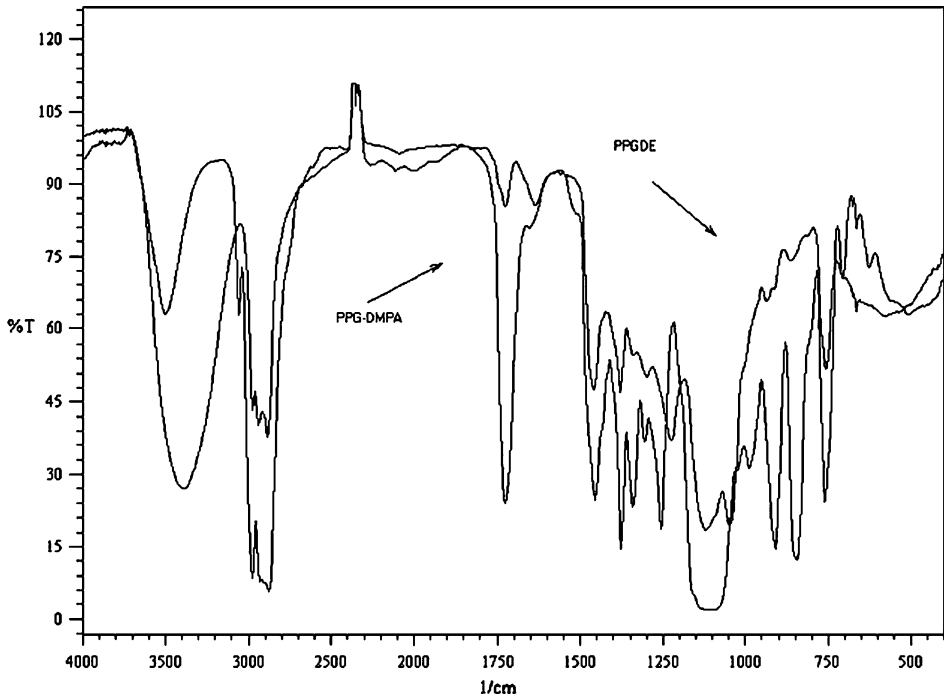


Fig. 1 FT-IR spectra of PPGDE and PPG–DMPA prepolymer

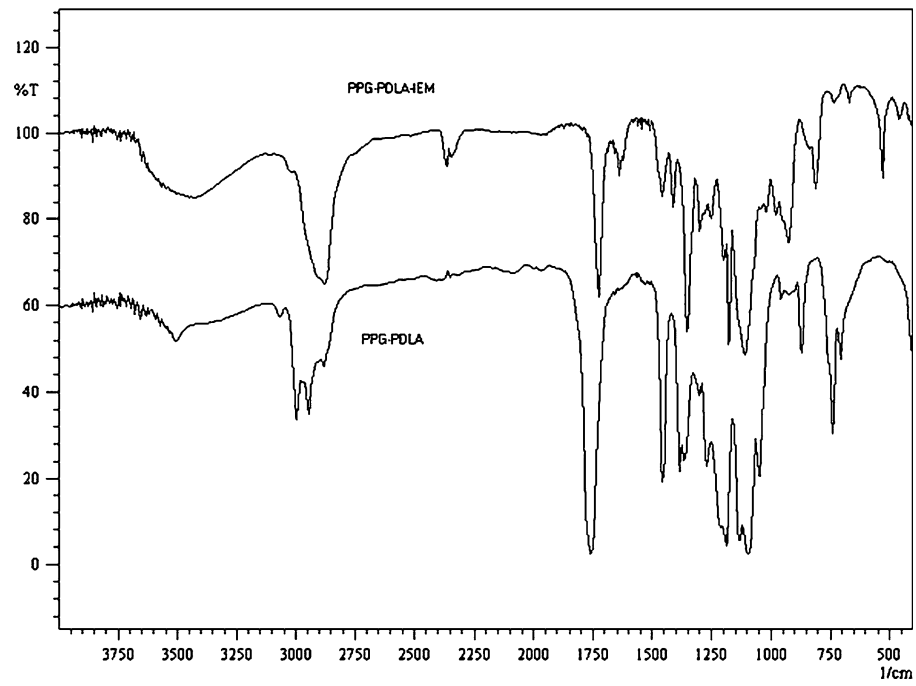


Fig. 2 FT-IR spectra of PPG–DMPA–PDLA and PPG–PDLA–IEM copolymers

imide band was formed at 1750 cm^{-1}. One can also see a split in the band at 1750 cm^{-1} belonging to carbonyl groups of acrylate and imidazole-functionalities.

CAB-381-20 was reacted with isocyanato ethyl methacrylate (IEM) in the presence of dibutyl tin dilaurate as a catalyst. As seen in Fig. 5, the complete disappearance of the absorption band at 2275 cm^{-1}, which is assigned to the characteristic isocyanate group, confirms the completion of the reaction between IEM and CAB. It also shows the characteristic =N–H stretching band at 3335 cm^{-1} and C=C double bond vibrations of methacrylate group at 1635 and 810 cm^{-1}.

Table 3 shows the gelation percentages and the equilibrium mass swelling results of the hydrogels. It can be seen in Table 3 that the PDLA–CAB–IEM-25-0 hydrogel has 89 wt% gel fraction. The gelation percentages

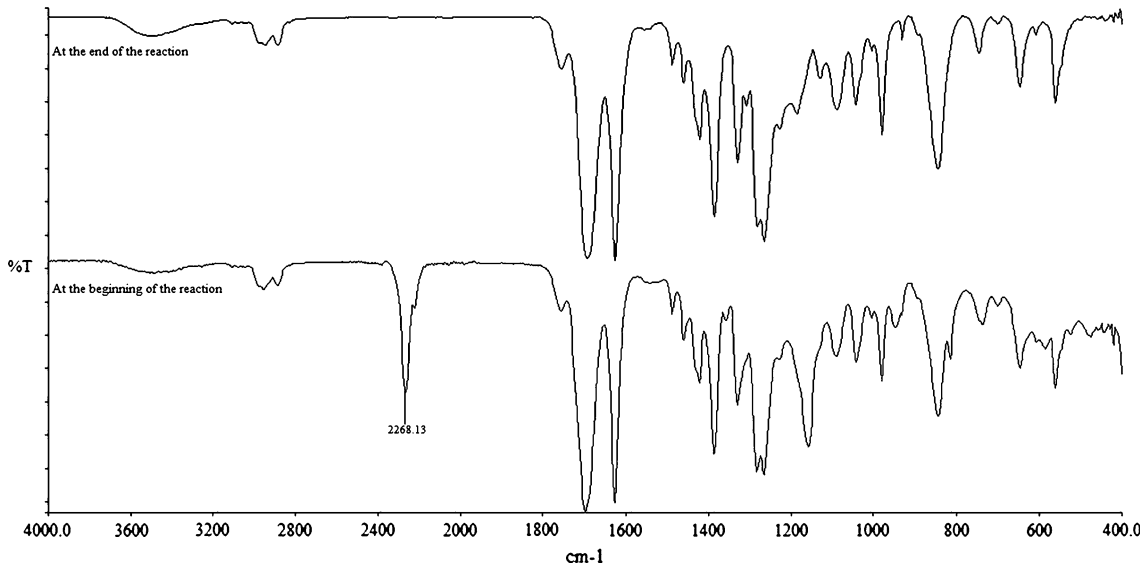


Fig. 3 FT-IR spectra of PPG–PDLA–IEM. Disappearance of –NCO peak at 2270 cm^{-1} confirms the reaction of the completion of the reaction between IEM and PPG–PDLA copolymer

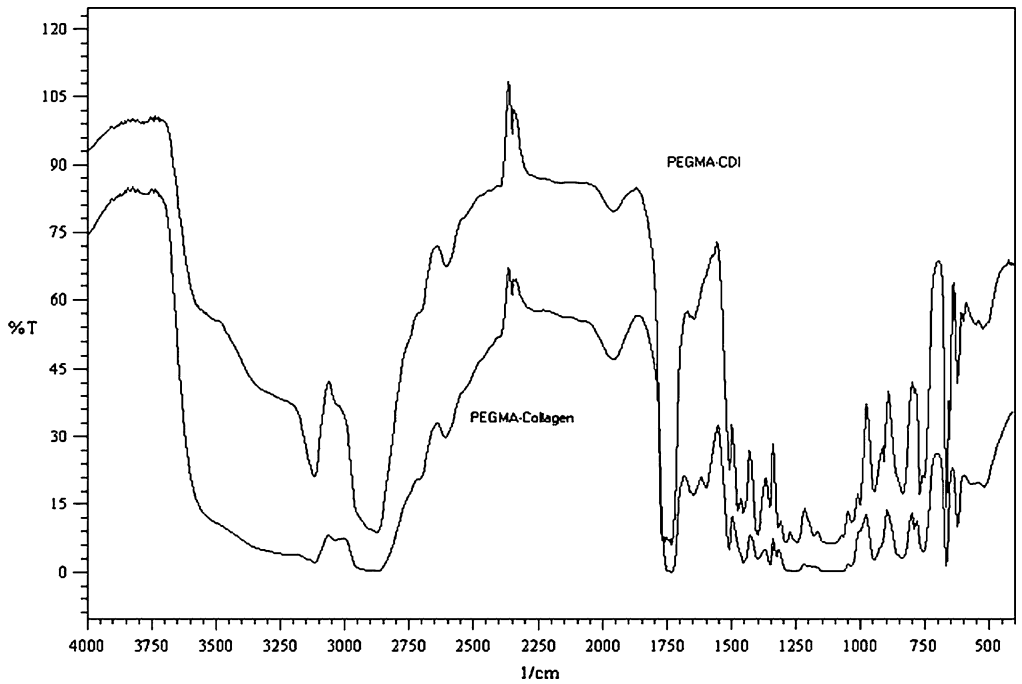


Fig. 4 FT-IR spectra of PEGMA–CDI and PEGMA–collagen

increased from 89 to 96 wt% with an increase in methacrylation ratio of CAB. This is due to the fact that the double bonds of CAB–IEM resin have an additional contribution to the crosslinking process. On the other hand with an increase in gelation percentage of the hydrogels, swelling decreases from 70 to 55%. It is also well known that the increase in crosslinking density of hydrogels makes an adverse effect on their swelling ratios [32].

Cell adhesion and proliferation are affected by the structure, chemical composition and especially hydrophilic property of the scaffold [33]. Hydrophilicity of the hydrogels containing different amount of PEGMA–collagen, was investigated using static water contact angle. In literature, it has been reported that hydrophobic polymers are unfavorable for cell attachment [34]. Cells attach and spread more easily on hydrophilic surfaces than hydrophobic

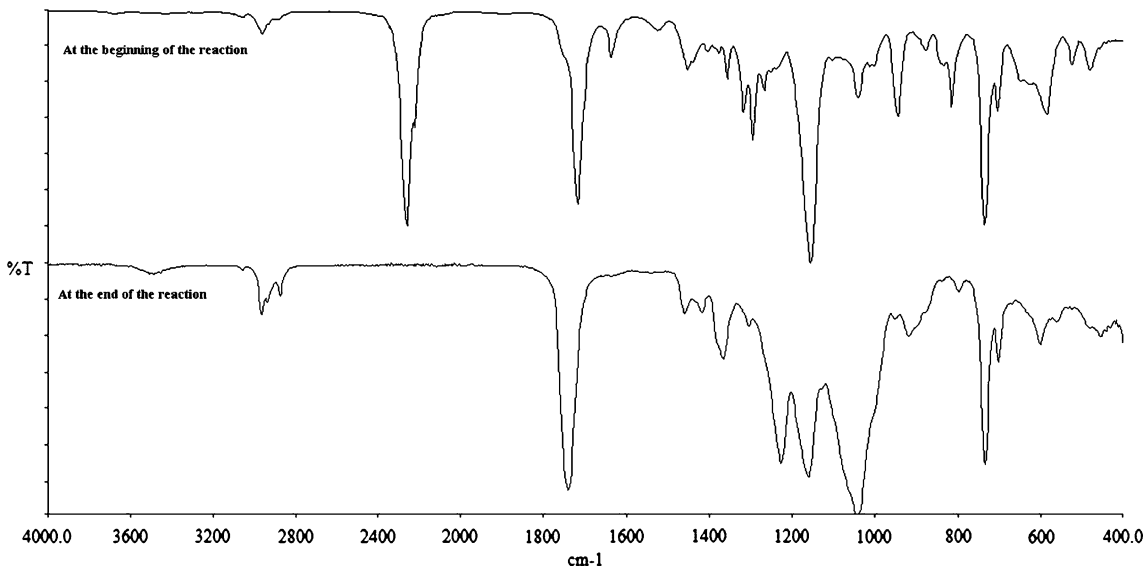


Fig. 5 FT-IR spectra of CAB–IEM. Disappearance of –NCO peak at 2270 cm^{-1} confirms the reaction of the completion of the reaction between IEM and CAB

Table 3 Characterization of the hydrogels

GEL	% Swelling	% Gelation
PDLA–CAB–IEM-25-0	70 ± 2.1	89 ± 1.2
PDLA–CAB–IEM-50-0	60 ± 1.7	93 ± 1.6
PDLA–CAB–IEM-75-0	55 ± 2.3	96 ± 2.1

Results are expressed as mean ± the standard deviation of the mean ($n = 3$)

Table 4 Water contact angles of the dry polymeric discs

Sample	PEGMA–collagen content (%)	Contact angle
PDLA–CAB–IEM 50	0	81 ± 0.8
PDLA–CAB–IEM 50	2	76 ± 1.2
PDLA–CAB–IEM 50	4	74 ± 0.7
PDLA–CAB–IEM 50	8	68 ± 1.9

Results are expressed as mean ± the standard deviation of the mean ($n = 5$)

surfaces [35]. Table 4 shows the contact angle results of the hydrogels. As can be seen in Table 4, the dry hydrogels, which were not containing PEGMA–collagen showed relatively high contact angle. Contact angle values measured on the dry hydrogels showed that, hydrophilicity of the surface increased with increasing PEGMA–collagen content.

The scaffolds used for tissue engineering applications are mostly solid, tough and brittle. Because of this, they can be broken or lose their dimensions during shaping procedure. Difficulties in shaping the scaffolds to fit into complicated or irregular-shaped defect sites and need for bigger surgical invasions are the main problems for solid scaffold applications. In contrast, injectable systems provide the advantages of mouldability to fill the gap between prosthesis and a bone or fill irregular bone cavities and defects completely, simple incorporation of bioactive factors, and limited surgical invasion [36]. Hence, the viscosity of the system becomes an important factor for such applications. The results of viscosity changes in uncured compositions by changing the modification ratio of CAB–IEM are shown in Table 5. The viscosities were found to increase from 1140 to 1460 cP as the modification ratio of the CAB–IEM increased from 25 to 75%. The viscosity results show that all formulations behave more like a Newtonian fluid and they are suitable for injectable systems.

Table 5 The viscosity of the hydrogel formulations

Gel	Speed (rpm)	Tork (%)	Viscosity (cp)
PDLA–CAB–IEM-25-0	20	47.5	1140 ± 20
PDLA–CAB–IEM-50-0	20	53	1365 ± 15
PDLA–CAB–IEM-75-0	20	43.5	1460 ± 20

Results are expressed as mean ± the standard deviation of the mean ($n = 3$)

The aim of using the degradable polymeric scaffolds is to implant the scaffold directly into the body for cell attachment. After implantation of the cells in the body, they cannot survive without efficient and adequate amount of oxygen and other nutrients. Once implanted, cells from surrounding living tissue attach to the scaffold to attain oxgen and other nutrients and form functional tissue [37]. After implantation, when the scaffold is populated with

cells and has gained the desired physical and mechanical properties the scaffold will degrade [5]. The scaffold's degradation rate should be adjusted to match the rate of tissue regeneration, so that it has disappeared completely once the tissue is repaired. Otherwise, removing scaffold will necessitate another surgical intervention. In literature degradation studies were performed as hydrolytic degradation in a desired medium, and enzymatic degradation in a natural environment [38, 39]. The degradation of the hydrogels was investigated by suspending them in phosphate buffer saline of 37°C at pH 7.4 and by measuring the weight loss over a 6-mount period. The in vitro degradation of hydrogels is shown in Fig. 6. The hydrogels degrade via hydrolysis of ester groups in the cross-links into low molecular weight degradation products. In all samples, there was an initial period of more rapid mass loss. The mass loss observed during the first few days is due to the sol fraction that is present in the system. In the CAB–IEM-75 based hydrogel sample, about 35% of the mass was lost in 175 days, while the weight loss of CAB–IEM-25 based hydrogel was 40% in the same time interval. This difference in mass loss is due to the methacrylation ratio of the CAB resin. The hydrogel prepared by the resin with low methacrylic content have lower crosslinking density. It has been generally considered that the degradation behaviour of the hydrogels depends on the crosslinking density. High crosslinking density retard the degradation of the hydrogel [40, 41]. This leads to easier and faster degradation of hydrogel.

Differential scanning photocalorimetry is a unique method for the fast and accurate evaluation of the photo reactivity of monomers [42]. The heat liberated during the polymerization reaction was directly proportional to the number of vinyl groups reacted in the system. By integrating the area under the exothermic peak, the conversion of the double bonds (DBC) or the extent of reaction could be determined according to the following formula [43]:

$$\mathrm{DBC} = \Delta H_t / \Delta H_o^{\mathrm{theor}} \quad (5)$$

where ΔH_t is the reaction heat evolved at time t, and $\Delta H_o^{\mathrm{theor}}$ is the theoretical heat for complete conversion. For acrylates H_0 is usually in the range of 78–86 kJ mol^{-1} and for methacrylates 56–58 kJ mol^{-1} [35]. The rate of polymerization (Rp) is directly related to the heat flow (dH/dt) by the following Eq. 5 [43]:

$$\mathrm{Rp} = \mathrm{d(DBC)}/\mathrm{dt} = (\mathrm{dH}/\mathrm{dt})/\Delta H_o^{\mathrm{theor}} \quad (6)$$

All the formulations investigated in this study photopolymerized very rapidly and became completely non-tacky after a few seconds. The photo-DSC data of the hydrogel formulations are shown in Figs. 7, 8. With an increasing methacrylation percentage, the [methacrylate]/[acrylate] ratio of the hydrogel increases. It is known that, acrylates have significantly higher Rp and lower t_{max} than methacrylic compounds [44]. As expected, the CAB–IEM-25 formulation with higher acrylate content gave higher

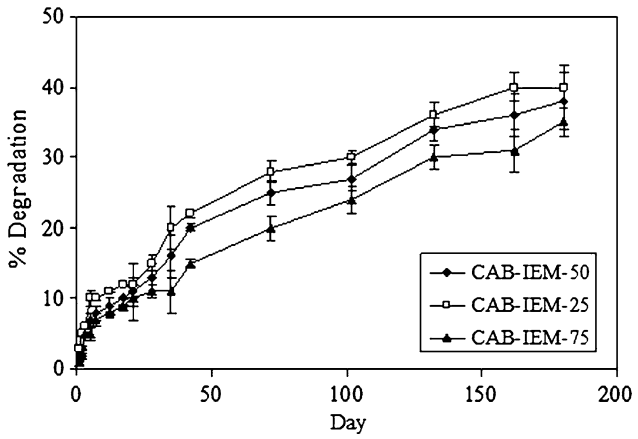


Fig. 6 The in vitro degradation kinetics of hydrogels. Error bars represent the standard deviation of the mean (results averaged over four measurements)

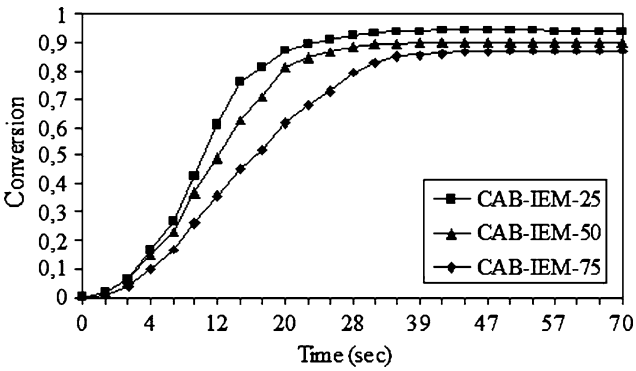


Fig. 7 The double bond conversion of the hydrogel formulations

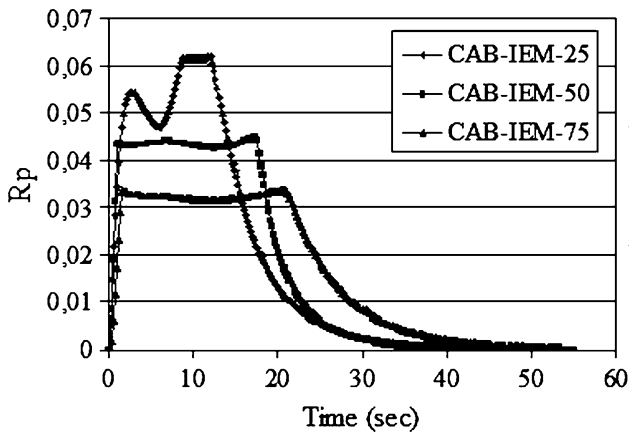


Fig. 8 The rate of the polymerization of the hydrogel formulations

Rp. Moreover, due to the higher double bond content of PDLA–CAB–IEM-75 formulation, the solidification of the polymer was attained faster. Therefore some of the entrapped double bonds in the dimensional structure stay unpolymerized. Therefore, the double bond conversion of the PDLA–CAB–IEM-75 based hydrogel was the lowest compared to the hydrogels with a lower methacrylation percentage.

Biocompatibility is the major property for the scaffold used for cell growth. As used herein while the term "biocompatible" refers generally to compatibility with living tissue or a living system, the term "nontoxic" generally refers to substances which, upon ingestion, inhalation, or absorption through the skin by a human or animal, do not cause, either acutely or chronically, damage to living tissue, impairment of the central nervous system, severe illness or death [45]. In that regard, cells can adhere and grow on biocompatible materials. In this study biocompatibility of the hydrogels and cell-hydrogel interactions were investigated with scanning electron microscopy (SEM). Collagen is the most widely found protein and a collagen molecule consists of three intertwined protein chains that form a helical structure. Collagen can be resorbed into body, is non-toxic, and produces only a minimal response. Because of these properties collagen exhibits excellent attachment and biological interaction with cells [46]. To investigate the effect of collagen on the cell adhesion hydrogels with 0–8 wt% collagen tethered poly (ethylene glycol) monoacrylate (PEGMA–collagen) were prepared. Even though the optimum structural results such as rate of polymerization and % conversion were obtained with highly methacrylated CAB resin (CAB–IEM-75%), the increasing degree of methacrylation makes the resin slightly soluble in the hydrogel formulation. Therefore, the CAB–IEM-50 resin was selected as the main structure of hydrogel formulation for the cell-hydrogel interaction studies. As seen clearly from Fig. 9, 3T3 mouse fibroblasts do not attach on the hydrogels having no collagen. The cell adhesion was significantly enhanced in the hydrogel with increasing amount of collagen (Figs. 10, 11). Also, the results shown in Fig. 11c state that, some of the cells have stated to divide. This is due to a more accessibility of the amino-groups of collagen, which is known for better cell adhesion. In addition, SEM investigations showed that the hydrogel without collagen has crater like (Fig. 9a–b) surface morphology. However, in case of collagen containing hydrogels, the cells fill these holes after cell seeding (Figs. 10, 11). This is attributed to the good adhesion and spreading of cells on the surface of the hydrogels. As mentioned above, collagen addition causes the good adhesion. On the other hand to investigate the cell viability with time, the HUVEC cells were seeded on hydrogels and kept in cell culture for 24, 48 and 72 h. According to the

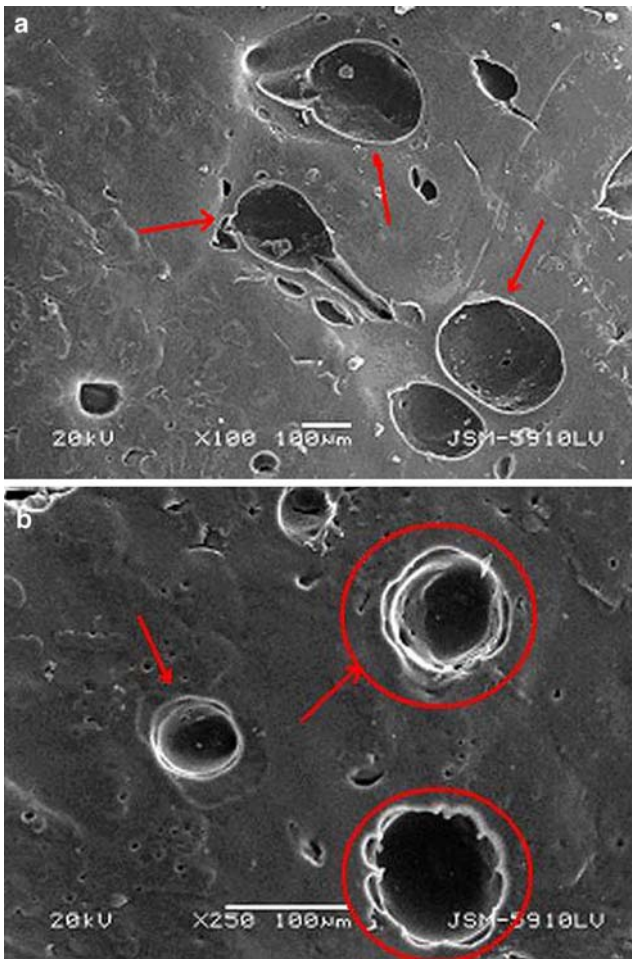


Fig. 9 SEM micrographs 3t3 fibroblasts cultured on the surface of PDLA–CAB–IEM-50 containing 0% collagen. **a** ×100 magnitude. **b** ×250 magnitude, 24 h following seeding

time dependent studies, the amount of cells on the hydrogel surface was slightly increased (Figs. 12, 13).This could mean that cells adhered with further multiplication and they were still living. For determining cell viability only MTT test was performed and Ref. [31] was given to support our conclusion.

The tetrazolium-based colorimetric assay (MTT test) is a quantitative assay, based on the reduction of yellow tetrazolium MTT by mitochondrial dehydrogenase enzyme from metabolically active cells into insoluble purple formazan crystals [47]. These formazan crystals are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells [48]. The number of living cells is directly proportional to the amount of the formazan crystal produced. The color can be quantified spectrophotometrically. MTT assay permits qualitative assessment of the cell's response after culture on PDLA–CAB–IEM-50 hydrogel.

Figure 14 showed that with the increasing amount of collagen in PDLA–CAB–IEM-50 based hydrogels, cell

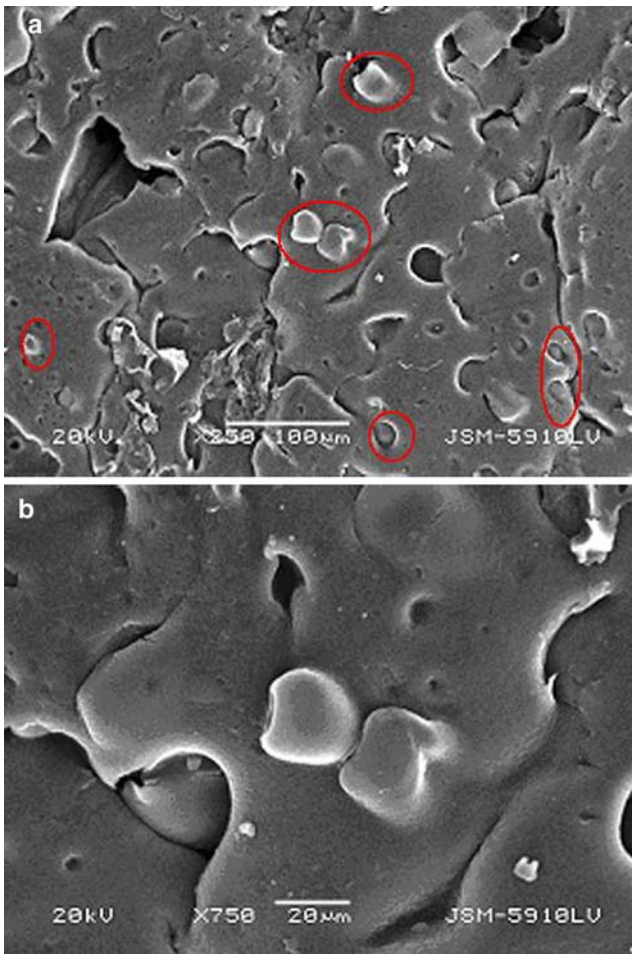


Fig. 10 SEM micrographs 3T3 fibroblasts cultured on the surface of PDLA–CAB–IEM-50 containing 4% collagen. **a** ×250 magnitude. **b** ×750 magnitude, 24 h following seeding

viability for both of cell lines increases too. The percentage of viable cells after 3 days of incubation in PDLA–CAB–IEM-50 based hydrogels containing 8% collagen was significantly (28% (ECV304, $P < 0.05$) and 30% (3T3, $P < 0.05$)) higher than the PDLA–CAB–IEM-50 based hydrogels without collagen. Therefore, the hydrogels having collagen were non-cytotoxic and supported cell growth. The scanning electron microscopy (SEM) investigations were in accordance with MTT results.

4 Conclusion

Hydroxyl end groups of cellulose acetate butyrate and previously synthesized poly(propylene glycol-co-lactic acid) were reacted with isocyanato ethyl methacrylate to achieve double bonds for photopolymerization. Collagen type I was attached to PEG–monoacrylate through carbodiimide chemistry for enhanced cell affinity of hydrogels. The structural properties of the hydrogels were changed by

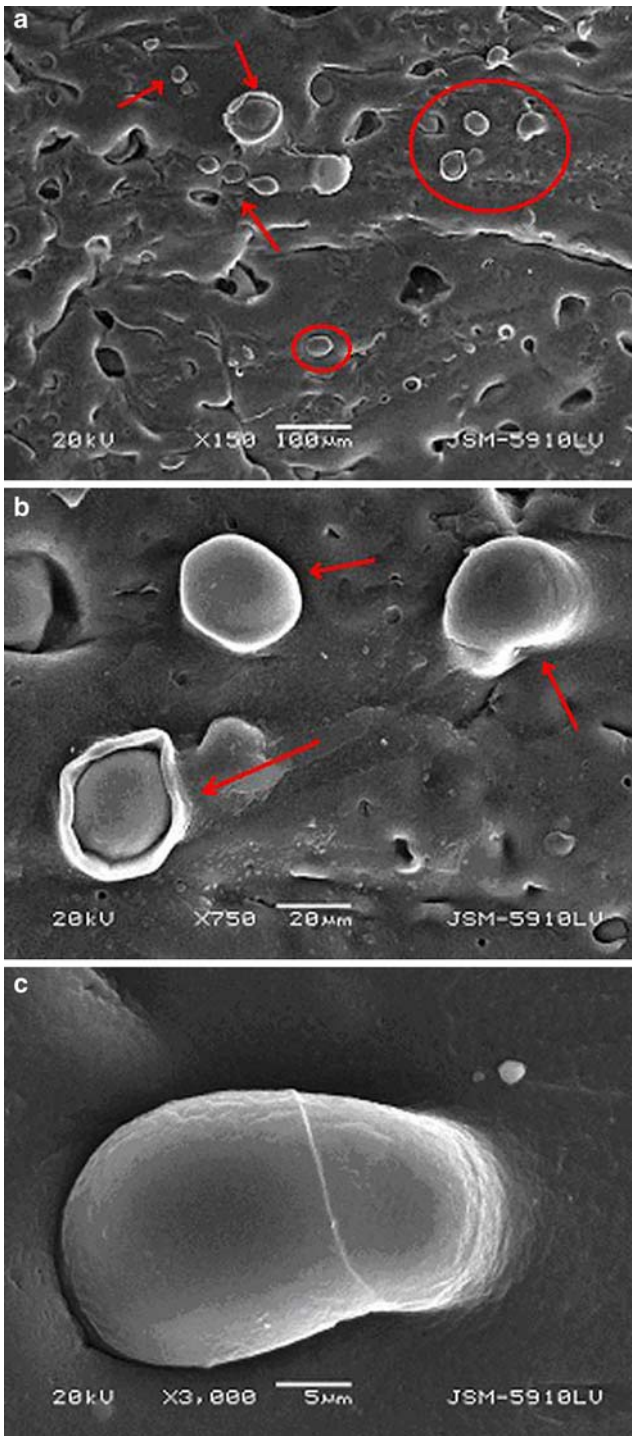


Fig. 11 SEM micrographs 3T3 fibroblasts cultured on the surface of PDLA–CAB–IEM-50 containing 8% collagen. **a** ×150 magnitude. **b** ×750 magnitude. **c** ×3000 magnitude, 24 h following seeding

the modification ratio of the CAB–IEM. The cell adhesion of the hydrogels was improved with the increasing content of collagen tethered PEG–monoacrylate (PEGMA–collagen). SEM images confirmed the cell adhesion on the hydrogel surface. MTT assay also confirmed that, after

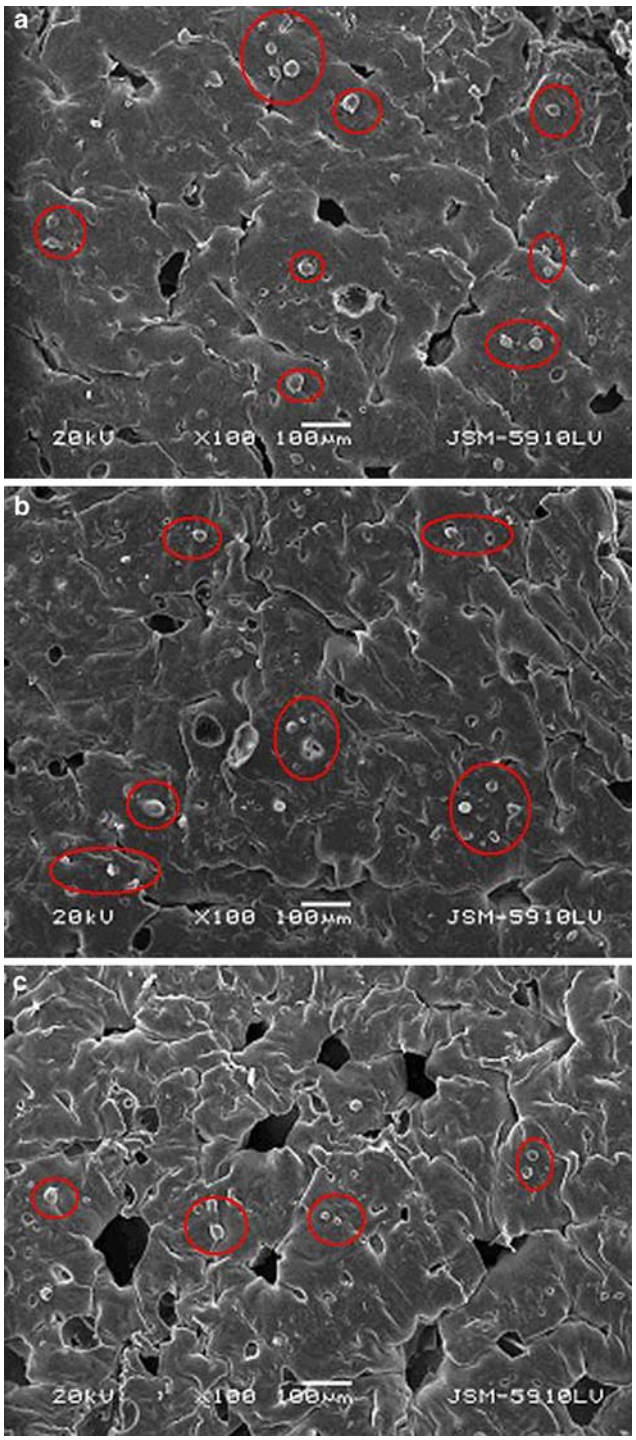


Fig. 12 SEM micrographs of HUVEC cells cultured on the surface of PDLA–CAB–IEM-50 containing 8% collagen. **a** 24 h, **b** 48 h, **c** 72 h following seeding

3 days of culture, cells maintain their cell activity and are still viable. The results suggest that PDLA–CAB–IEM-50 based hydrogel scaffolds with collagen are good candidates for tissue engineering applications.

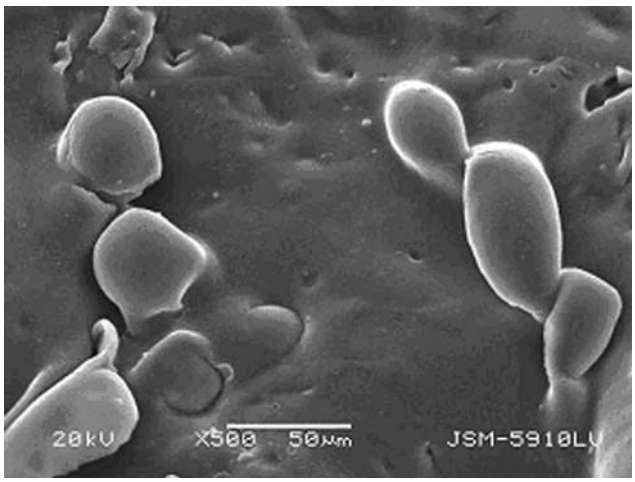


Fig. 13 SEM micrograph of HUVEC cells cultured on the surface of PDLA–CAB–IEM-50 containing 8% collagen with ×500 magnitude

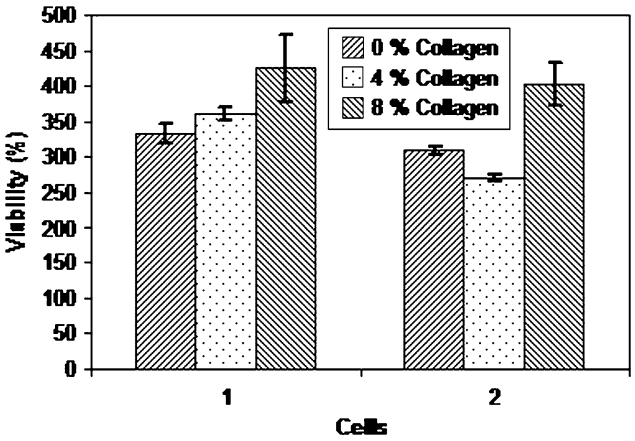


Fig. 14 The % viability of ECV304 HUVEC and 3T3 fibroblast cells. Error bars represent the standard deviation of the mean (results averaged over three measurements)

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