***In situ* forming hydrogels based on polyethylene glycol itaconate for tissue engineering application**

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Abstract. Novel strategies have been proposed to enhance the quality of surgery by scheming noninvasive methods. For this reason, photo-curable *in situ* forming hydrogels have been well developed during advancements in the regenerative medicine. In this study, polyethylene glycol itaconate (PEGI) was synthesized by reacting polyethylene glycol (PEG) with different molecular weights (1000, 4000 and 8000 g mol⁻¹) and itaconyl chloride. The synthesized PEGIs were fully characterized and employed as a macromonomer for the preparation of *in situ* forming hydrogels using a combination of camphorquinone and dimethylaminoethyl methacrylate as a reactive photoinitiator system, and hydroxyethyl methacrylate as a reactive diluent. The physical properties of the hydrogels including gel yield, equilibrium swelling and compressive strength were determined. The hydrogel based on PEG 4000 with a gel yield of 86%, a water uptake of 103%, a compressive modulus of 11.2 MPa, an elongation at break of 9% and a curing time of 4 min was selected for the encapsulation of rabbit articular chondrocyte cells. The cytocompatibility of the *in situ* formed hydrogels was evaluated using 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, live-dead fluorescence assays and optical microscopy observations. Glycosaminoglycans were quantified by dimethylmethylene blue staining from the encapsulated chondrocytes after 14 days. The proposed *in situ* forming hydrogel can be considered as an injectable and photocurable carrier for cell delivery in cartilage tissue engineering.

Keywords. Polyethylene glycol itaconate; chondrocyte; *in situ* forming; hydrogel; tissue engineering.

1. Introduction

Various therapeutic approaches have been explored for tissue regeneration by using different kinds of biomaterials. Among all, the most favourable one is that requires the least possible invasiveness for surgery [1,2]. In some cases, the defect site requires filling that injection of a biocompatible *in situ* curable substance can be an ideal solution to meet these needs [3]. These structures also provide additional advantages, including exactly filling the defect with no supplemental shaping and enhancement in adjacent tissue attachment or mechanical interlocking [4,5]. Hydrogels have been the material of choice for many applications in regenerative medicine due to their desirable physical and biological characteristics [6,7]. Polyethylene glycol (PEG) has been extensively investigated for use in the preparation of hydrogels due to its availability, simplicity in structure and ability for modifications [8,9]. Moreover, this synthetic polymer can be functionalized to produce three-dimensional (3D) networks [10,11]. One way is to conjugate unsaturated groups such as double bond to the

PEG structure [12,13]. By applying suitable polymerization conditions on the embedded double bond, simultaneous polymerization and 3D network formation as well as encapsulation of cells or bioactive agents can be performed [14]. Among different polymerization strategies, photopolymerization has attracted much attention. Nowadays, this method has a wide range of biomedical applications such as dental restoration, implant fixation and *in situ* formation of carriers for drug delivery or cell transplantation [4,15–17].

The camphorquinone (CQ)/tertiary amine photoinitiation system is widely used to prepare *in situ* forming structures for the applications such as dental restorative resins [18–21]. The light curing mechanism of the CQ/amine system has been described previously by Teshima *et al* [22]. This process is triggered by the absorption of visible light, excitation of CQ and production of free radicals through the abstraction of a hydrogen atom from the tertiary amine. These amine primary radicals attack the carbon double bonds on the main chains and provide free sites for further crosslinking reactions.

Unsaturated functional groups, such as maleimides, acrylates or fumarate, can be utilized for the introduction of double bonds to PEG chains [23–27]. Itaconic acid with a carbon–carbon double bond (C=C) within its side chain may become a choice for the PEG functionalization in light curing application. The copolymers of PEG and itaconic acid were recently suggested for drug delivery application [28,29]. In our previous study, polyesterification of low molecular weight polycaprolactone diols was successfully performed with itaconyl chloride (IC) [30]. This paper reports on the design and synthesis of light-curable hydrogels based on PEG itaconate (PEGI). The physical–chemical characteristics and cytotoxicity of these compositions were evaluated for tissue engineering application.

2. Experimental

2.1 Materials and methods

PEG with different molecular weights (1000, 4000 and 8000 g mol^{−1}), IC, *N*-vinylpyrrolidone, toluene, propylene oxide, methylene chloride and diethyl ether were purchased from Merck (Germany). Hydroxyethyl methacrylate (HEMA), dimethylaminoethyl methacrylate (DMAEMA) and CQ were purchased from Sigma-Aldrich (USA).

2.2 Synthesis of PEGI

PEG was pre-dried in a vacuum oven at 70°C for 24 h and then dried *via* azeotropic distillation using dried toluene under a nitrogen atmosphere before use. A dark three-neck round-bottom flask equipped with a nitrogen inlet, a thermometer and an ice bath was charged with dried PEG (10 mmol), propylene oxide (25 mmol) and dried methylene chloride (20 ml). IC (10 mmol) dissolved in dried methylene chloride (10 ml) was added drop-wisely to the flask over 3 h. After this, the reaction mixture was stirred at room temperature for 48 h under a nitrogen atmosphere. Finally, methylene chloride was removed in a rotatory vacuum evaporator and the residue was precipitated with diethyl ether. The obtained PEGI was washed twice with diethyl ether and dried in a vacuum oven at room temperature for 24 h.

2.3 Hydrogel preparation

A solution of PEGI (1 g) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Scotland, 1 ml) was prepared. Specified amounts of CQ and DMAEMA were also dissolved in HEMA as shown in table 1. The curing process was initiated by the irradiation of CQ–DMAEMA–HEMA solution with blue light (Litex 695 LED curing light, intensity of 1200 mW cm^{−2}, λ_{max} of 470 nm) for 2 min. Then, PEGI solution was added, the mixture was irradiated again by blue light for 6 min.

Table 1. The formulations used for the preparation of hydrogels.

Hydrogel	PEGI	HEMA (ml)	CQ (mg)	DMAEMA (μl)
H1	PEGI1000	1	2.5	2.5
H2	PEGI1000	1	5	5
H3	PEGI1000	1	10	10
H4	PEGI4000	1	2.5	2.5
H5	PEGI4000	1	5	5
H6	PEGI4000	1	10	10
H7	PEGI8000	1	2.5	2.5
H8	PEGI8000	1	5	5
H9	PEGI8000	1	10	10
H10	—	1	2.5	2.5
H11	—	1	5	5
H12	—	1	10	10
H13	PEGI4000	0.5	5	5
H14	PEGI4000	2	5	5

2.4 Instruments

Gel permeation chromatography (Agilent 1100, PLgel 10 μm) was employed to determine the molecular mass of the synthesized PEGI using tetrahydrofuran as the eluent with a rate of 1 ml min^{−1}. Fourier transformed infrared (FTIR) spectroscopy was performed on a Nicolet spectrometer (model 6700) over the wavenumber of 400–4000 cm^{−1} at a resolution of 2 cm^{−1} using KBr pellets. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker apparatus (model AMX-300).

2.5 Methods

The gelation time of the hydrogels was measured *via* the test tube inverting method [31] at 25°C in a water bath. For this purpose, a test tube containing 1 ml of each formulation was irradiated and the fluidity was observed by tilting the tube every 30 s. The time of ceasing the fluidity was recorded as the gelation time.

To determine the gel yield of the prepared hydrogels, samples were soaked initially in water at 50°C for 20 min and then in phosphate buffer saline (PBS) at 25°C for 24 h to remove non-reacted substances. The specimens were then dried in a vacuum oven to reach a constant weight. Gel yield (%) was calculated based on the weights of the dried sample and the mass of initial monomers (PEGI and HEMA):

$$\text{Gel yield} = \frac{W_{\text{dried sample}}}{W_{\text{initial monomers}}} \times 100. \quad (1)$$

Water uptake of the prepared hydrogels was determined according to ASTM D570 *via* immersing samples in PBS at 37°C and weighing regularly at different time intervals to obtain a constant weight. Water uptake was calculated

according to the following equation:

$$\text{Water uptake} = \frac{W_{\text{wet sample}} - W_{\text{initial mass}}}{W_{\text{initial mass}}} \times 100. \quad (2)$$

In order to assess the percentage of degradation, the samples were immersed in PBS at 37°C for 3 months and every three days, medium was replaced with fresh PBS. At the end of the experiment, the samples were soaked in PBS at 25°C for 24 h and dried in a vacuum oven.

$$\text{Gel yield after 3 months} = \frac{W_{\text{after 3 months}}}{W_{\text{initial monomers}}} \times 100, \quad (3)$$

$$\text{Degradation after 3 months} = \text{Gel yield 24 h} - \text{Gel yield 3 months}. \quad (4)$$

2.6 Chondrocyte isolation

Rabbit articular (white New Zealand) cartilage slices were isolated from lateral femoral condyle, predigested with trypsin (0.25%, Sigma-Aldrich, USA) for 1 h and digested with collagenase type II (0.08 mg ml⁻¹, Sigma-Aldrich, USA) for 12 h at 37°C [32]. The cells were transferred to DMEM/Ham's F12 culture medium supplemented with 10% fetal calf serum (Nanobioarray, Iran).

2.7 Cell encapsulation

PEGI solution was prepared in DMEM/Ham's F12 culture medium and sterilized by a filter (0.4 µm). Cells (3 × 10⁵) suspended in the culture medium was added to the PEGI solution. HEMA-CQ-DMAEMA solution was separately prepared and irradiated by blue light for 2 min in order to activate the curing process. Then, PEGI-cell solution was added and the mixture was irradiated again by blue light for 6 min to fulfil the curing process. Finally, the culture medium was added to the cell loaded hydrogels following the incubation at 37°C in a humidified atmosphere with 5% CO₂.

The fluorescent dead-live procedure was performed on encapsulated chondrocytes using propidium iodide (PI, Sigma-Aldrich, USA, 750 µM)–acridine orange (AO, Sigma-Aldrich, USA, 670 µM) double staining [31]. Culture medium and dimethyl sulfoxide (Sigma-Aldrich, USA, 10%) were used as negative and positive controls, respectively.

The cells were also fixed by Karnovsky fixation solution and dehydrated in graded alcohols according to a previously reported procedure [32]. The specimens were sputter-coated with gold and viewed by scanning electron microscopy (SEM, Tescan VegaII, Czech).

2.8 Agar diffusion cytotoxicity evaluation

Cells were seeded at a density of 5 × 10³ cell per well in 24 well plates and incubated at 37°C for 24 h. A solution of agar in deionized water (1%) was autoclaved and added to each

well (250 µl) to cover the cells. Subsequently, cylindrical hydrogels (diameter of 4 mm and length of 8 mm) were placed on the solidified agar following the addition of culture medium and incubation at 37°C for 7 days. After this, medium, hydrogel and agar gel were removed and the toxicity of each sample was quantified by the addition of 250 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) solution in PBS (0.5 mg ml⁻¹) to each well. After 4 h, the purple formazan crystals were dissolved in isopropanol (250 µl, Merck, Germany) by incubating at 37°C for 15 min. The absorbance of each well was measured via a multiwell UV–Visible spectrometer (Shimadzu, model 1601) at 575 nm and normalized by the control measurement.

2.9 Determination of glycosaminoglycan (GAG) expression

Dimethyl methylene blue (DMMB, Sigma-Aldrich, USA) was used for the quantification of GAGs expressed by the encapsulated chondrocyte according to a previously reported procedure [31]. Cells (3 × 10⁴, after 14 days of isolation and 2nd passage) were encapsulated and cultured in 1 ml of culture medium for 14 days by adding another 1 ml of the medium at day 7. The amount of 500 µl of the medium of each well was added to 1.5 ml of ice-cold acetone (Merck, Germany) and stored at –20°C for 24 h. The samples were centrifuged at 1800 rpm, 4°C for 30 min. The supernatant was removed and a papain solution (20 µg ml⁻¹) activated with cysteine (5 mM) was added. The mixture was maintained at 60°C for 16 h and boiled for 15 min. The presence of GAGs in each sample was revealed by the addition of the DMMB reagent. The optical density was immediately measured at 545 nm using a microplate reader (Awareness Technologies, model Stat Fax 2100, USA). A working range of a known concentration of chondroitin sulphate C (shark cartilage extract, Sigma-Aldrich, USA) was used as the standard solution.

3. Results and discussion

3.1 Synthesis and characterization of hydrogels

PEGIs were synthesized from the polycondensation reaction of PEG with different molecular weights (1000, 4000 and 8000 g mol⁻¹) and IC (figure 1a). The polyesterification reaction was studied by FTIR spectroscopy (figure 2a). The peaks at 1728 and 1098 cm⁻¹ for PEGI1000 confirmed the formation of ester bonds between PEG and IC [33]. In addition, the peak at 1644 cm⁻¹ showed the presence of the double bond in the structure of PEGI1000. The molecular structure of the synthesized PEGIs was completely investigated via NMR spectroscopy (figure 2b). The protons of the methylene (CH₂) group belong to the double bond appeared as two peaks at 5.6 and 6.2 ppm. The peaks at 3.2 and 3.4–3.6 ppm

Figure 1. (a) Reaction pathway for the synthesis of PEGI. (b) Preparation procedure of cell encapsulated PEGI hydrogel.

Figure 2. (a) FTIR and (b) ^1H -NMR spectra of PEG1000.

were related to the methylene portions within IC and PEG moieties, respectively [34]. The average molecular weights of PEG1000, PEG4000 and PEG8000 were 1400, 4500 and 8800 g mol^{-1} , respectively.

For the preparation of hydrogels, HEMA was used as a reactive diluent and CQ/DMAEMA as a reactive photoinitiator system. HEMA-based polymers have been well established as biomaterials for different clinical applications

such as contact lens or bone cement [35,36]. Cytotoxicity evaluation of the aqueous extract of three different dental based materials showed the lowest toxicity for HEMA in comparison with glycerol dimethacrylate and triethylene glycol dimethacrylate [37]. The inhibitory effect of water at low concentrations (<5%) on CQ/DMAEMA has been reported previously [38]. However, this problem can be solved by the addition of aqueous part (culture medium and cells) after the activation of the photoinitiator system. For this purpose, the HEMA–CQ–DMAEMA solution was initially pre-cured *via* irradiating by blue light for 2 min and then the PEGI–DMEM solution (with or without the cells) was added followed by irradiation for 6 min (figure 1b). Another advantage for the pre-curing treatment is the reduction in cell toxicity risks emerged from the CQ/DMAEMA system. Several studies examined the hazardous effects of this system on the generation of reactive oxygen species and oxidative DNA damage [39]. It was proposed that oxidative DNA damage produced by the irradiated CQ/tertiary amine system could be significantly diminished by employing antioxidant substances such as glutathione and *N*-acetyl-L-cysteine [40].

The results of the curing test indicated unsuccessful curing of the H1, H2, H4 and H7 formulations. Moreover, increasing the amount of the initiator/activator improved the compression strength of the hydrogels (table 2). Although the number of free radicals increases at higher initiator/activator concentrations, some of them participate in a self-reacting process instead of the crosslinking reaction. These unreacted chemicals may leach out from the hydrogels and affect the biocompatibility of the final implant. Therefore, from the cytotoxicity viewpoint, these concentrations should be optimized to the lowest range. Conversely, no gelation was observed at a lower amount of the initiator/activator (the ratio of CQ/DMAEM to HEMA was 0.05%) even at a longer curing time. The surplus amount of the initiator leads to the

Table 2. Physical and mechanical properties of the hydrogels.

Sample	Gel time (min)	Gel yield (%)	Water uptake (%)	Gel yield (%)	Degradation after 3 months (%)	Compressive strength (MPa)	Elongation (%)
H1	>30	—	—	—	—	—	—
H2	>30	—	—	—	—	—	—
H3	13	63.5	114 ± 6	58.3	5.2	0.98	8.4
H4	>30	—	—	—	—	—	—
H5	4	86.5	102 ± 9	76.6	9.9	11.2	9.1
H6	3.5	91.8	108 ± 4	81.1	10.7	9.6	10.4
H7	>30	—	—	—	—	—	—
H8	4	84.7	87 ± 11	77.0	7.7	10.4	11.5
H9	3	89.0	90 ± 5	81.4	7.6	8.5	11.8
H10	3	69.6	108 ± 7	59.2	10.4	24.1	23.7
H11	2.5	81.5	96 ± 4	78.3	3.2	39.8	50
H12	2.5	83.4	87 ± 6	79.7	3.7	26.5	46.25
H13	6	61.2	108 ± 6	37.1	24.1	2.51	3.3
H14	1.5	64.6	76 ± 5	59.8	4.8	23.1	42.8

Figure 3. Rabbit chondrocytes encapsulated in different PEGI hydrogels: The cells cultured on the tissue culture plate as (a) control, (b, g) H3, (c, h) H5, (d, i) H8, (e) H13 and (f) H14. The bars are 20 μ m.

stabilization of free radicals before activation of double bonds. This raises the chance for remaining unreacted residues which may act as a plasticizer [41]. In this investigation, samples H6,

H9 and H12 with the extra quantity of CQ demonstrated lower strength than their corresponding samples H5, H8 and H11, respectively.

Figure 4. Live/dead assay with AO/PI staining of samples: (a) negative control, (b) positive control, (c) H3, (d) H5, (e) H10 and (f) H12. The bars are 20 µm. SEM images of (g) H3, (h) H5 and (i) H13. (j) The viability of isolated chondrocytes using agar diffusion assay after 7 days culture. (k) Quantification of GAG released from encapsulated chondrocytes in different hydrogels.

The viscosity and opacity of the formulations were increased during the light-curing process. A higher amount of CQ/DMAEMA resulted in faster gelation. The yield of gelation for H6, H7, H9 and H10 was more than 80% (table 2). It seems that the longer curing time and higher amount of HEMA are required to complete the gelation of PEG1000 based hydrogels. The incorporation of PEGIs decreased the compression strength of the hydrogels. The lower molecular weight of PEG1000 chains, in comparison with PEG4000 and PEG8000, reduced the compression strength of the final products (table 2). From the other point of view, entanglement in PEG4000 and PEG8000 maintained the longer chains in the structure after washing and swelling processes. Samples based on PEG8000 showed higher elongation at break and lower compressive strength compared to the corresponding PEG4000-based samples. The presence of water molecules in PEG1 solution acts as a retardant for the crosslinking reaction [38]. Therefore, the highest compression strength and elongation were obtained in the absence of PEGIs.

In the next step, the ratio of HEMA to PEG4000 was altered (H5, H3 and H14). The compressive strength of the samples was improved with increasing the amount of HEMA. In contrast, the absorption of equilibrium water decreased with increasing the amount of HEMA. However, due to the cytotoxicity of HEMA, the lowest possible amount of HEMA is preferable.

3.2 Cell encapsulation in hydrogels

The photomicrographs of the viable rabbit chondrocytes encapsulated in PEG1 hydrogels (H3, H5, H8, H13 and H14) are depicted in figure 3. The spherical morphology of these cells at the first week of isolation after culturing on tissue culture polystyrene plate (TCPS) is shown in figure 3a.

The live and dead cells encapsulated in PEG1 hydrogels after AO/PI staining are shown in figure 4a–f. The scanning electron micrographs of the isolated rabbit chondrocytes encapsulated in different samples are observed in figure 4g–i. The morphology of chondrocytes in these samples was spherical, where chondrocyte-specific phenotype maintained. Figure 4j displays the results of the cytotoxicity assay in comparison with the control. This chart demonstrated that H3, H5, H6, H8, H9, H13 and H14 have cell viability more than 80%. The exposure of the cells to hydrogel extracts revealed that the toxicity is increased using a higher amount of HEMA. Furthermore, elimination of PEG1 in the samples (H10, H11 and H12) increases the toxicity of the specimens. H5, H8 and H13 with the lowest amount of HEMA showed lower cytotoxicity compared to the other samples. Figure 4k shows the comparison of GAG released from rabbit chondrocytes after 14 days of encapsulation. H5 and H6 represented a higher amount of GAG expression and confirm the positive influence on chondrocyte proliferation in comparison with control (TCPS) and other samples.

4. Conclusion

In this study, PEGIs were synthesized and used in the formulation of photocurable hydrogels using the CQ/DMAEMA system as a photoinitiator and HEMA as a reactive diluent. The physical, chemical and biological properties of the hydrogels were characterized and optimized for cell encapsulation. The hydrogel based on PEG4000 with a gel yield of 86%, a water uptake of 103%, a compressive modulus of 11.2 MPa, an elongation at a break of 9% and a curing time of 4 min was selected for the encapsulation of rabbit articular chondrocyte cells. The viability of encapsulated chondrocytes was evaluated by MTT and live/dead assays and the cell morphology was visualized by SEM. Moreover, the function of the encapsulated chondrocytes was determined by quantification of proteoglycan release. The results revealed that this hydrogel can be utilized as a scaffold for articular cartilage regeneration. Future investigations were conducted on stem cell differentiation and in animals.

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